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A Study of the Glycosides in the Hot Water Extract of the Green Bark of *Populus trichocarpa*

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A STUDY OF THE GLYCOSIDES IN THE HOT WATER EXTRACT OF THE GREEN BARK OF *Populus trichocarpa*

A thesis submitted by

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SUMMARY

Two 4000-g. samples of green bark from Populus trichocarpa were extracted with hot water. The hot water extract, which represented 14% of the original bark, was given successive exhaustive extractions with chloroform, ethyl ether, and ethyl acetate. These extractions removed 8, 10, and 20% respectively of the hot water extract. Portions of each extract were chromatographed on polyamide columns before and after mild lead subacetate treatments, and the aqueous eluates were examined for glycosides and related materials.

Silica gel thin-layer chromatograms sprayed with 50% sulfuric acid and paper chromatograms sprayed with diazotized p-nitroaniline, silver-sodium hydroxide, and other sprays were used for the initial detection of materials. Semiquantitative determinations were made on each isolated material. These determinations were obtained from the total weight of material obtained from a specific peak of a polyamide column chromatogram and from the quantity of crystals isolated.

The chloroform extract contained salicyl alcohol, pyrocatechol, and a large amount of uninvestigated fatty material. After lead subacetate treatment, salicin and a trace of tremuloidin were obtained. The salicin, salicyl alcohol, and pyrocatechol each represented 4% of the chloroform extract.

The ethyl ether extract contained 35% trichocarpin, 5% salireposide, 5% p-coumaric acid and 7% of a new glycoside – trichocarposide. After lead subacetate treatment, the same quantity of each of these materials was detected.

In a structural study of trichocarposide, a mild alkaline hydrolysis gave only p-coumaric acid and salicin. A carbon and hydrogen analysis indicated that the empirical formula of trichocarposide was $C_{22}H_{24}O_9$. Periodate oxidations indicated that the p-coumaroyl group was on the alpha or six position of salicin while an enzymatic hydrolysis showed that it was not probable that the p-coumaroyl

group was on the alpha position. Thus, trichocarposide is 6-O-p-coumaroyl salicin [o-hydroxymethylphenyl 6-O-(p-hydroxycinnamoyl)-β-D-glucopyranoside].

About 2% of the ethyl acetate extract was salicin and after lead subacetate treatment, 9% of the extract was salicin. After a mild acid hydrolysis of part of the ethyl acetate extract, a trace of unidentified material, which gave salicin and salicylic acid after mild alkaline hydrolysis, was obtained. This unidentified material, m.p. 173-5°, had an IR spectrum showing two carbonyl groups.

It appears that most of the salicin exists in the bark combined in an unidentified mixture of materials. This unidentified mixture is not the same as that found in Populus tremuloides or P. grandidentata since large amounts of salicyloyl tremuloidin were not obtained after hydrolyses, and this unidentified mixture was found in the ethyl acetate extract rather than in the chloroform or ethyl ether extract.

In addition, there were large amounts of unidentified materials in the ethyl acetate extract. One of these was isolated as a crude material, which after mild alkaline hydrolysis and acidification, gave p-coumaric acid. The finding of this p-coumaroyl compound plus trichocarposide and free p-coumaric acid explains the large amount of p-coumaric acid which was previously found after an alkaline hydrolysis of the bark of Populus trichocarpa.

The aqueous raffinate from the ethyl acetate extraction contained glucose, fructose, sucrose, and a trace of arabinose. Large amounts of uninvestigated materials also were present in the raffinate.

Many other materials from all the fractions were noted by thin-layer and paper chromatography but were not isolated as crystalline compounds.

INTRODUCTION

In a preliminary evaluation by Pearl, et al. (1) on the green bark of Populus trichocarpa (northern black cottonwood), crystals of two glycosides, salicin and salireposide, and two aglucones, salicyl alcohol, the aglucone of salicin and other related glycosides, and cis-1,2-cyclohexanediol (2), the aglucone of grandidentatin, were obtained from a hot water extract which was treated with lead subacetate. These materials have been found in the bark of other Populus species (1-6), but not in the brown bark of Populus trichocarpa (7). Pearl and Darling (5, 8) showed that the classical lead subacetate treatment used for "clarification" of the hot water extract causes some acyl migration as well as a possible hydrolysis of glycosides.

Thieme (9) has shown that a chromatographic separation with a polyamide column may be used instead of the clarification with lead subacetate. Thieme and co-workers have used polyamide column chromatography to separate many glycosides (including salicin and salireposide) from the hot water extracts of various European Salix species (9-13).

In a study of fungistatic materials, Butin and Loeschcke (14) studied the hot water extract of one-year winter twig bark from Populus trichocarpa finding two fungistatic materials; pyrocatechol and a new glycoside, trichocarpin. Later, Loeschcke and Francksen (15) identified the structure of trichocarpin (Fig. 1). Trichocarpin has been found also in Populus balsamifera (16).

In this investigation, the glycosides in the green bark of Populus trichocarpa were studied extensively. The hot water extract of this important pulpwood species was given successive exhaustive chloroform, ethyl ether, and ethyl acetate extractions instead of the initial lead subacetate treatment. Polyamide column chromatography was used to separate the extractives. Polyamide chromatography was used also

on portions of extracts, which were treated with lead subacetate. Thin-layer and paper chromatography were used for the initial detection of materials.

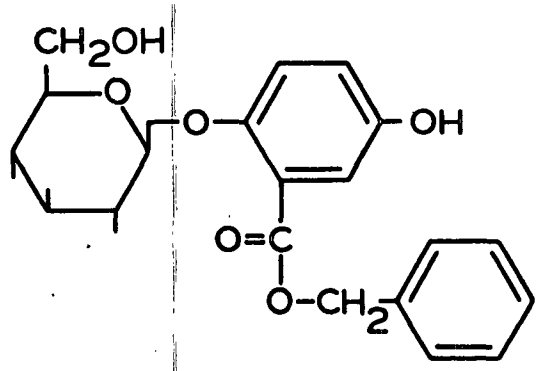

Figure 1. Trichocarpin

EXPERIMENTAL PROCEDURE

EXTRACTIONS

BARKS

Two samples of green bark of Populus trichocarpa were used in this study. The first sample (Bark 1), which was used for the initial studies, was obtained from Simpson-Lee Timber Company, Everett, Washington in October, 1959. It was chipped, dried, Wiley milled, bagged, and stored at room temperature. The moisture content was 5.4%.

The second sample (Bark 2), which was used for most of this investigation, was obtained from Crown Zellerbach Company, Camas, Washington. It was cut during the first week of March, 1965, barked by a hydraulic barker, and shipped to The Institute of Paper Chemistry. After air drying for a week, the bark was sorted into young, smooth, green and mature, furrowed, brown barks. The green bark was cut, Wiley milled, air dried for a week, and bagged. About 4000 g. of ovendry green bark were obtained.

HOT WATER EXTRACTION

The simple hot water extraction procedure described by Pearl, et al. (1, 6) was used on 1500 g. batches of bark. In this procedure, 1500 g. (ovendry weight) of bark were added to 11 liters of boiling water. The mixture was heated with stirring for one hour and then filtered through a cloth. The residual bark retained by the cloth was reextracted by an identical procedure. The resulting 20-22 liters of turbid solution were mixed with Celite, filtered through a Celite pad, and concentrated in a circulating evaporator to about 2 liters.

A double batch of Bark 1 and three batches of Bark 2 were processed. Twelve percent of ovendry Bark 1 and sixteen percent of ovendry Bark 2 were extracted by this procedure. In one batch of Bark 2, the first hot water extraction removed 12% of the material and the second extraction removed 4%.

Each batch of hot water extract was given successive exhaustive extractions with chloroform, ethyl ether, and ethyl acetate. The flow diagram for the general processing of the bark is shown in Fig. 2.

CHLOROFORM EXTRACTION

Each batch of hot water extract was centrifuged and filtered to remove solid materials. Then the liquid was extracted exhaustively with chloroform in a rotating ball extractor[1]. The chloroform extraction removed 8% of the hot water extract or about 1% of the original bark. Emulsion problems during the extraction were controlled by periodically removing the solid materials. Thin-layer chromatography (TLC) indicated that the materials removed at the beginning of the extraction were very similar to those removed at the end.

ETHYL ETHER EXTRACTION

Each batch of chloroform raffinate was concentrated in a rotary evaporator to remove residual chloroform, centrifuged to remove solid materials, and extracted with ethyl ether in a two-liter, liquid-liquid, nonagitated extractor. Exhaustive extraction of the chloroform raffinate removed 10% of the hot water extract. Emulsion problems during these extractions necessitated a very slow rate of extraction and hence long extraction times. The emulsion was broken periodically by siphoning it from the extractor and then concentrating it in a rotary evaporator.

[1]Manufactured by Rinco Instrument Co., Greenville, Illinois.

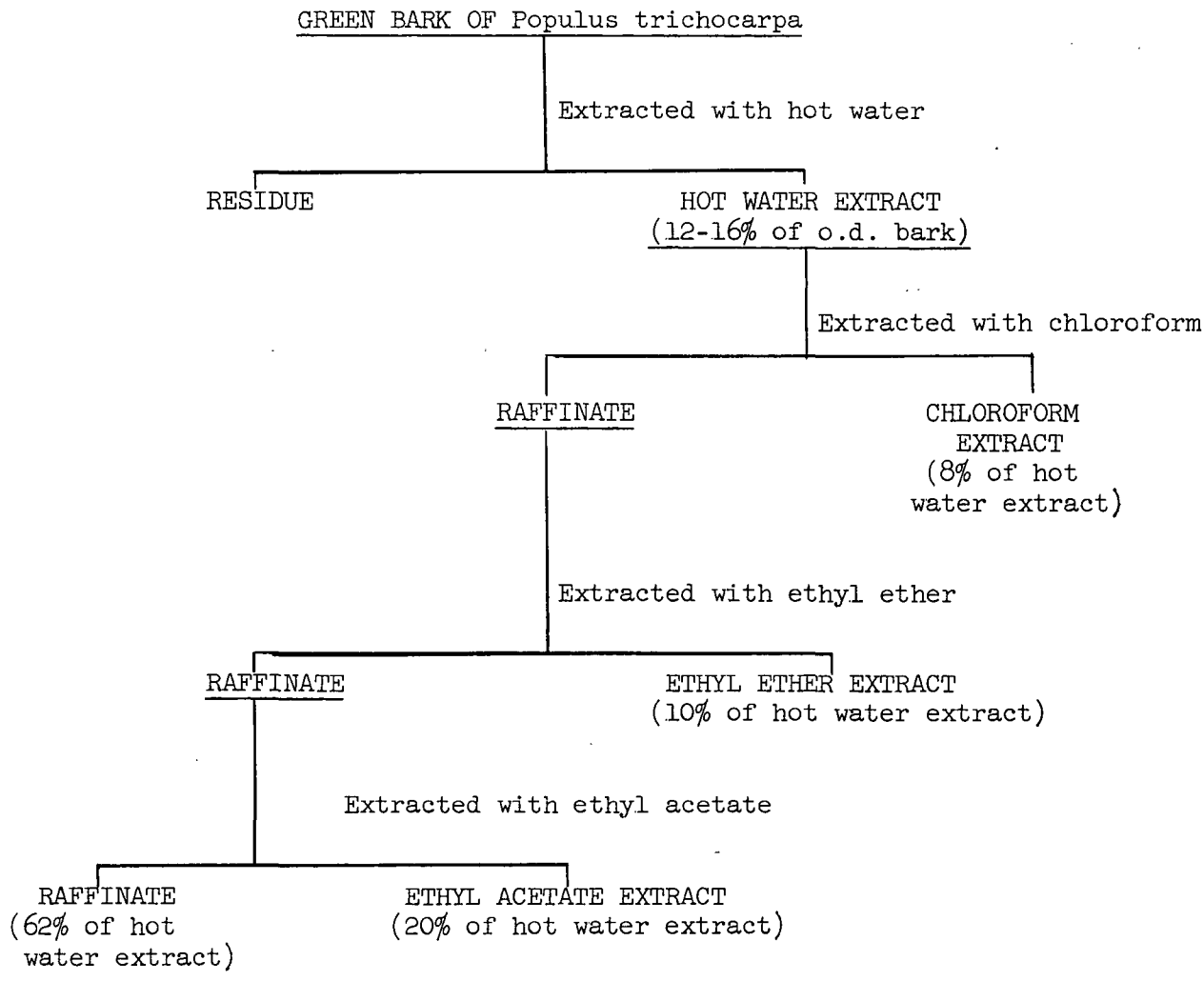

Figure 2. Extractions of Bark

ETHYL ACETATE EXTRACTION

The ethyl ether raffinate was concentrated in a rotary evaporator to remove traces of ether and then extracted exhaustively with ethyl acetate in the same manner as the ethyl ether extraction. Emulsions were not a problem in these extractions. The ethyl acetate extractions removed 20% of the hot water extract.

RAFFINATE

The ethyl acetate raffinate (Raffinate) was covered with toluene and kept under refrigeration until used. More than 62% of the original hot water extract remained in the raffinate.

CHROMATOGRAPHY

POLYAMIDE COLUMN CHROMATOGRAPHY

Throughout this study, chromatography was used for separation and preliminary identification of the glycosides and other materials. The separation of the extractives by means of elution chromatography on a polyamide column was one of the techniques extensively used. The following procedures were used for all polyamide column chromatograms. The polyamide[2] was extracted with warm methanol, washed with water, and poured into columns (9, 16). The polyamide was allowed to settle and the water was drained slowly from the columns. The columns were washed with several volumes of water. From two to twenty grams of material were evaporated to dryness, slurried in 20-100 ml. of warm water, and transferred to columns. The columns were eluted with 20-30 liters of water. The first 250 ml.

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[2]Polyamide was manufactured by M. Woelm Company, Eschwege, Germany. MN polyamide, made by Macherey, Nagel, and Co., Germany, also was used, but did not separate the extractives.

of eluate were collected as 25-ml. fractions and the next 750 ml., as 50 or 100-ml. fractions. After one liter of eluate was obtained, 500-ml. fractions were collected and after ten liters, 1 to 5-liter fractions were collected. The large fractions were evaporated under reduced pressure in a circulating evaporator and the 50-ml. and smaller fractions were concentrated in a rotary evaporator. Many of the fractions were evaporated to dryness. Others were concentrated and allowed to stand at room temperature or under refrigeration. Those fractions which were evaporated to dryness were dissolved in various solvents in an attempt to crystallize some of the materials.

Finally, some of the columns were eluted with methanol or ethanol. This removed 5-50% of the materials put on the column. Some materials were always retained by the column. These materials darkened with time, especially when exposed to light. These permanently adsorbed materials did not seem to have a significant effect on the chromatographic ability of the polyamide when reused in other columns.

Weight determinations of the eluted fractions provided one method of evaluating the effectiveness of the columns. The weights were determined by evaporating the eluate to dryness in a rotary evaporator and weighing the material to the nearest milligram. The weight of an individual fraction was divided by the volume of the fraction and plotted versus the amount of material eluted through the column to give an elution diagram.

These elution diagrams provided a semiquantitative determination of the maximum amount of a given constituent (16). Integration of the area of a peak gives the weight of materials in that peak. These weights are presented in the summary tables. When the peak weight is divided by the amount of material removed from the column, a maximum percentage for the given material is obtained. Correspondingly, the weight of crystalline material obtained from the same peak represents

a minimum weight. When this weight is divided by the amount of material put on the column, a minimum percentage is obtained. These percentages are adjusted so that all the values are based on the total extract.

This semiquantitative procedure is accurate for sharp, pure peaks. TLC and paper chromatography results were used to estimate the appropriate peak weights for indistinct peaks. A portion of almost every fraction obtained from the columns was spotted on paper and thin-layer chromatograms.

THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography (TCL) was used to detect glycosides. Glass plates (10 x 20 cm.) were coated with a 28% silica gel G solution using a Camag[3] coater. After air drying, the plates were activated by heating at 130° for 15 minutes and then stored in a desiccator. The plates were spotted with four to ten unknown materials and a known which usually consisted of salicin, salireposide, and trichocarpin. Then the plates were developed by ascension of the developer in a closed jar.

The developer was 4:1 chloroform – methanol with 5 ml. of acetic acid per liter of developer (8). The R_f of the materials was very dependent upon the equilibrium and temperature of the tank. In order to have consistent data, all R_f values were adjusted so that the R_f of trichocarpin was 0.45. This adjustment was made by either rerunning the chromatogram or making minor linear corrections.

After developing for 30 minutes, the plates were allowed to dry and then were inspected for visible and ultraviolet fluorescent spots. Trichocarpin and many of the unknown materials had fluorescent spots, while most of the other known

[3]Manufactured by Camag, Switzerland.

materials did not. Then the plates were given a light spray with 50% sulfuric acid and placed in a 105° oven for 10-15 minutes. Using this technique, glycosides and other phenolic materials were detected. Table I gives the R_f and color of some known materials.

PAPER CHROMATOGRAPHY

Paper chromatography was used for the preliminary identification of some materials. Whatman no. 1 papers were used in six different developers: 10:3:3 butanol – pyridine – water (BPW), 8:2:1 ethyl acetate – pyridine – water (EPW), 10:4:1 butyl formate – formic acid – water (BFW), benzene saturated with formic acid (BFA), butanol saturated with 2% aqueous ammonia (BA), and water with 1% acetic acid (water). These papers were sprayed with diazotized p-nitroaniline followed by sodium carbonate (DPNA) to detect phenolic materials (17), dipped with silver-sodium hydroxide (silver) to detect most glycol-containing materials (3), sprayed with p-anisidine hydrochloride (anisidine) to detect aldoses (18), sprayed with urea-phosphate (urea) to detect ketoses (19), sprayed with a 0.1% aqueous solution of Eastman dye 1954 [4-(4-dimethylamino-1-naphthylazo)-3-methoxybenzenesulfonic acid] adjusted with sodium hydroxide to pH 8.9 (indicator) to detect benzoic acid and other acidic materials (20), and inspected for ultraviolet fluorescence (UV) and UV after exposure to ammonia vapors. Almost every fraction was spotted on paper, developed (usually with water), and sprayed with DPNA and often silver.

The water developer was chosen because it effectively separated phenolic acids as well as other phenolic materials, because it separated cis and trans isomers of cinnamic acid derivatives (21, 22), and because reliable results were obtained in two to four hours. Table II gives some of the results of the water developer.

TABLE I

TLC RESULTS OF KNOWN MATERIALS

Material	R_f[a]	Color[b]
Glucose	0.05	Black
Salirepin	0.18	Orange
Salicin	0.27	Strong pink
Salireposide	0.40	Strong yellow orange
Grandidentatin	0.44	Weak yellow gray (weak UV)
Trichocarpin	0.45	Strong green black (blue UV)
Trichocarposide[c]	0.46	Weak gray pink
Salicyloyl salicin	0.54	Pink (blue UV)
Tremuloidin	0.55	Pink
Salicyloyl tremuloidin	0.65	Pink (blue UV)
Pyrocatechol	0.78	Purple
Salicyl alcohol	0.80	Strong pink
cis-1,2-Cyclohexandiol	0.80	Brown
p-Coumaric acid	0.85	Gray pink

[a] R_f in 4:1 chloroform – methanol.

[b] Color after spraying with 50% sulfuric acid and heating from 10 to 15 minutes at 105°.

[c] A new glucoside described in this study.

TABLE II

PAPER CHROMATOGRAPHIC RESULTS OF KNOWN MATERIALS

Material	R_f [a]	UV	NH_3+UV	DPNA	DPNA+UV
Quercetin	0.00	Brown	Yellow	Yellow	Brown
Querciturone	0.13	-Purple	Brown	Yellow	Brown
trans-Ferulic acid	0.30	Blue	Blue	Blue	Blue
trans-*p*-Coumaric acid	0.37	-Purple	Deep-blue	Blue	Blue
Trichocarposide	0.45(0.65)	–	Deep-blue	Weak-blue	–
Syringic acid	0.47	–	–	Blue	–
Vanillic acid	0.51	–	–	Purple	–
Acetosyringone	0.53	–	Deep-blue	Yellow	–
p-Hydroxybenzoic acid	0.58	–	–	Red	–
Acetovanillone	0.59	–	Deep-blue	Purple	Deep-blue
Gentisic acid	0.60	Blue	Blue	Yellow	Yellow-green
Syringaldehyde	0.60	–	-Purple	Yellow	–
Trichocarpin	0.61	Blue	Blue	Blue-purple	–
Vanillin	0.63	–	-Purple	Purple	–
Salireposide	0.67	–	–	Blue	–
Salicyloyl salicin	0.68	Blue	Deep-blue	Orange	–
cis *p*-Coumaric acid	0.71	–	–	Blue	–
Trichocarpigenin	0.74	Blue	Blue	Gray-blue	Blue
Pyrocatechol	0.76	–	–	Purple-green	–
Gentisyl alcohol	0.77	–	–	Yellow	–
Salicyl alcohol	0.78	–	–	Pink-red	–
Grandidentatin	0.79	–	Green-blue	Gray-blue	Green-blue
Salicin	0.88	–	–	none [b]	–
Glucose	0.93	–	–	none [b]	–

[a] R_f in water with 1% acetic acid.

[b] Spot not detected with DPNA, but detected with silver.

EXPERIMENTAL RESULTS

ETHYL ETHER EXTRACT

INTRODUCTION

The largest amount of glycosides was found in the ethyl ether extract. The extract was investigated by chromatography with polyamide columns. Part of the extract was treated with lead subacetate and subsequently investigated. A flow chart of these investigations is shown in Fig. 3.

POLYAMIDE COLUMN CHROMATOGRAPHY

The ethyl ether extract from Bark 2 was chromatographed directly on several polyamide columns (Fig. 3). The weight of materials eluted from one of these columns is shown in Fig. 4 and 5, the TLC results are given in Fig. 6, and a summary is presented in Table III. Figures 4 and 5 which are constructed from the weight of every other fraction, show four peaks. The fractions indicated by the first peak were composed of salicin (Fig. 7) and several other materials. The salicin was detected by TLC[4]. The fractions which contained salicin represent less than 2% of the extract. Salicyl alcohol (Fig. 8) and pyrocatechol (Fig. 9) were in fractions found between the first and second peak. Salicyl alcohol was identified by its DPNA red spot on paper chromatograms developed in water, BPW, and BA[5]. Pyrocatechol was identified by its TLC spot, as well as a characteristic blue-gray spot on paper chromatograms developed in water, BPW, and BA[6]. Several minor constituents were detected by TLC in the fractions shown between the first and second peak.

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[4]Salicin has been obtained as crystals from other extracts of this bark.

[5]An IR spectrum was obtained for salicyl alcohol from another extract of this bark.

[6]An IR spectrum was obtained for pyrocatechol from another extract of this bark.

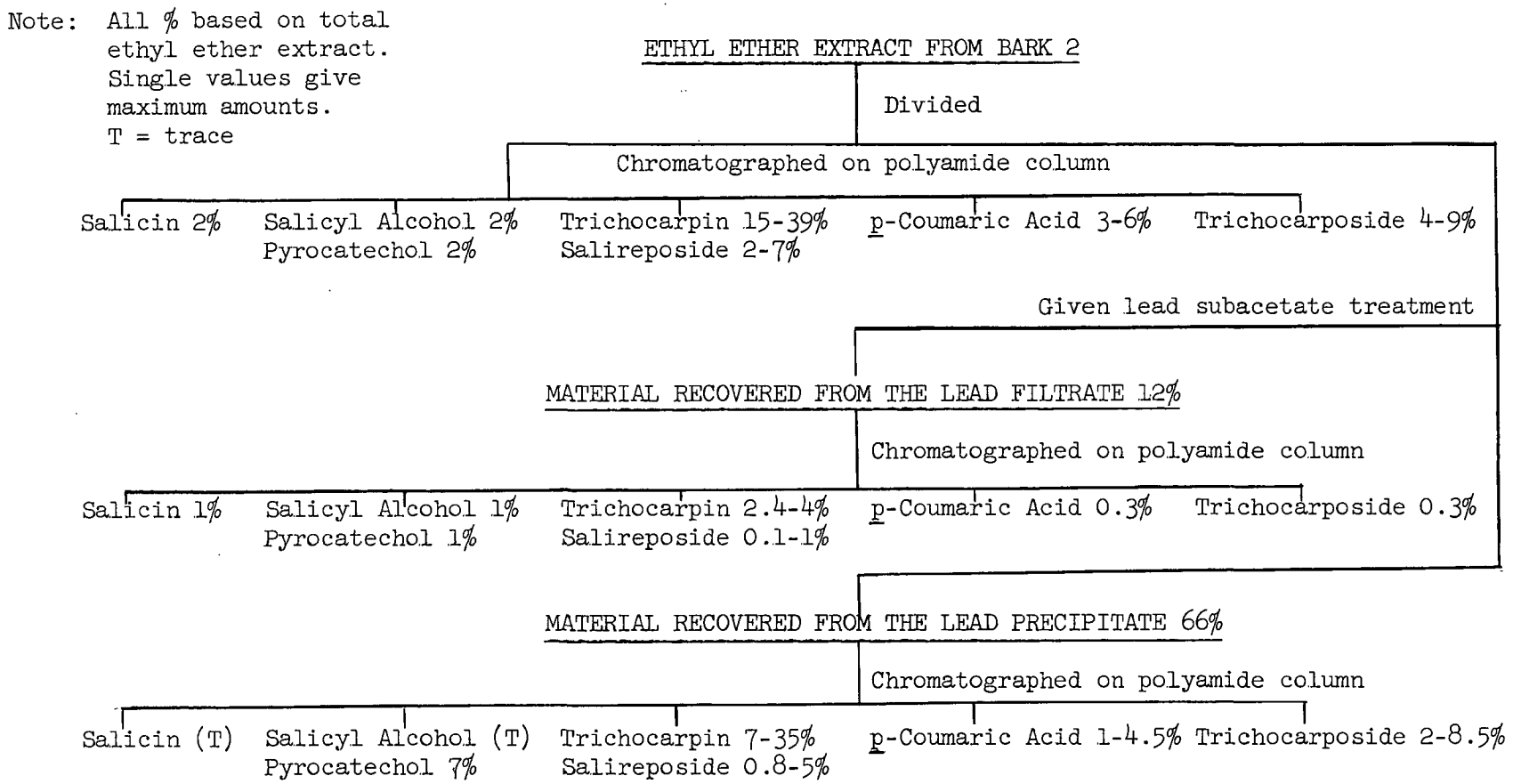

Figure 3. Flow Chart of Processing of Ethyl Ether Extract

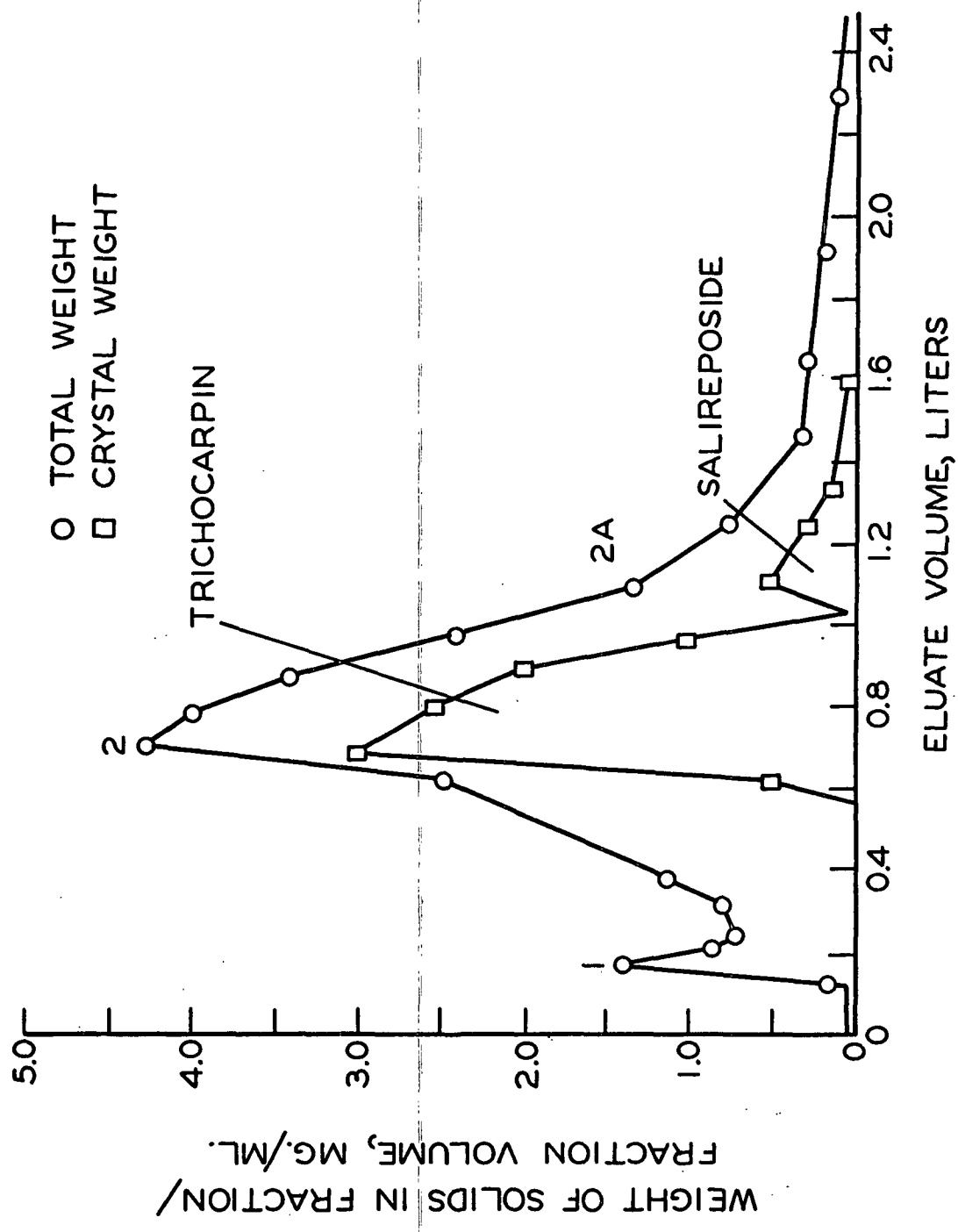

Figure 4. Elution Diagram of Polyamide Column Chromatography of Ethyl Ether Extract

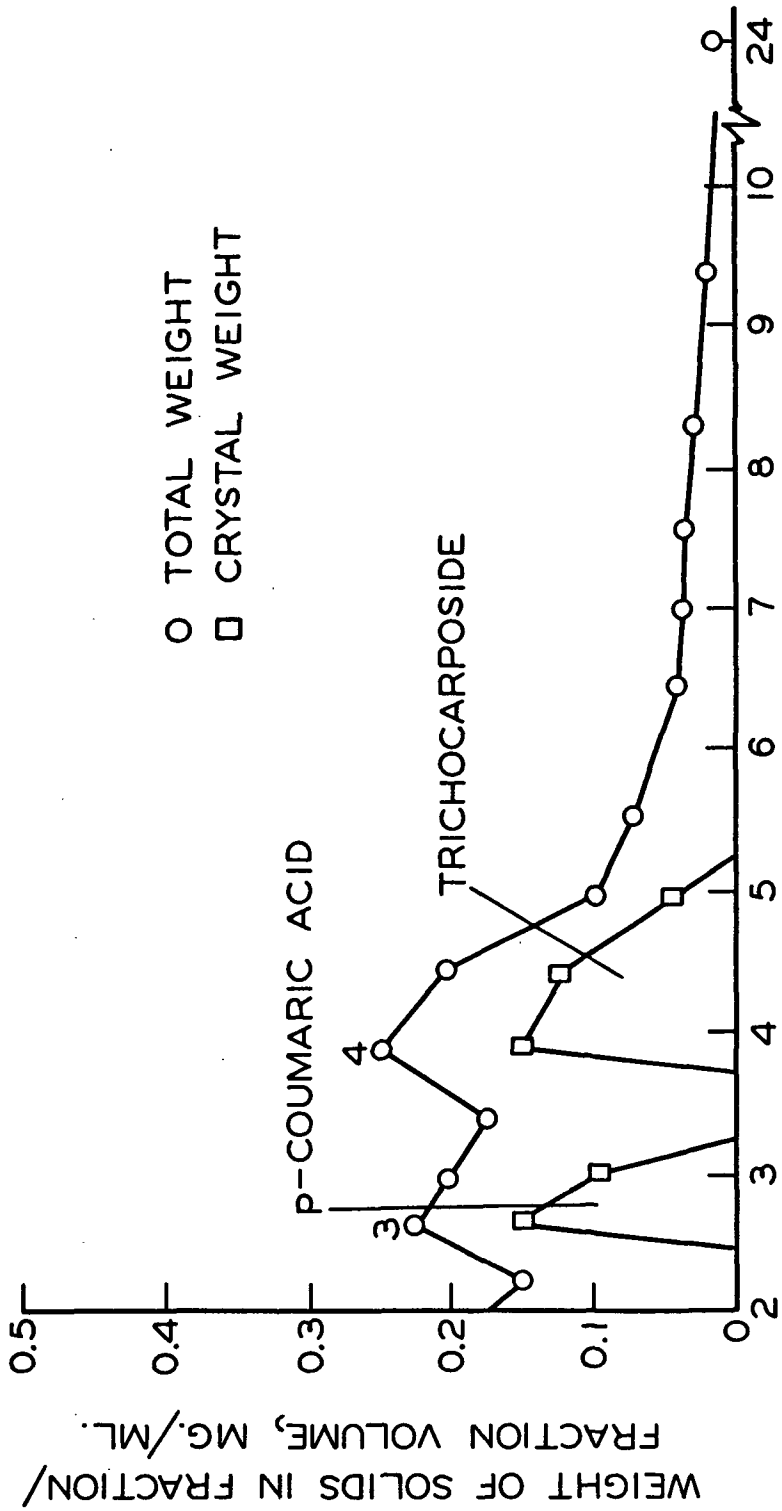

Figure 5. Elution Diagram of Polyamide Column Chromatography of Ethyl Ether Extract

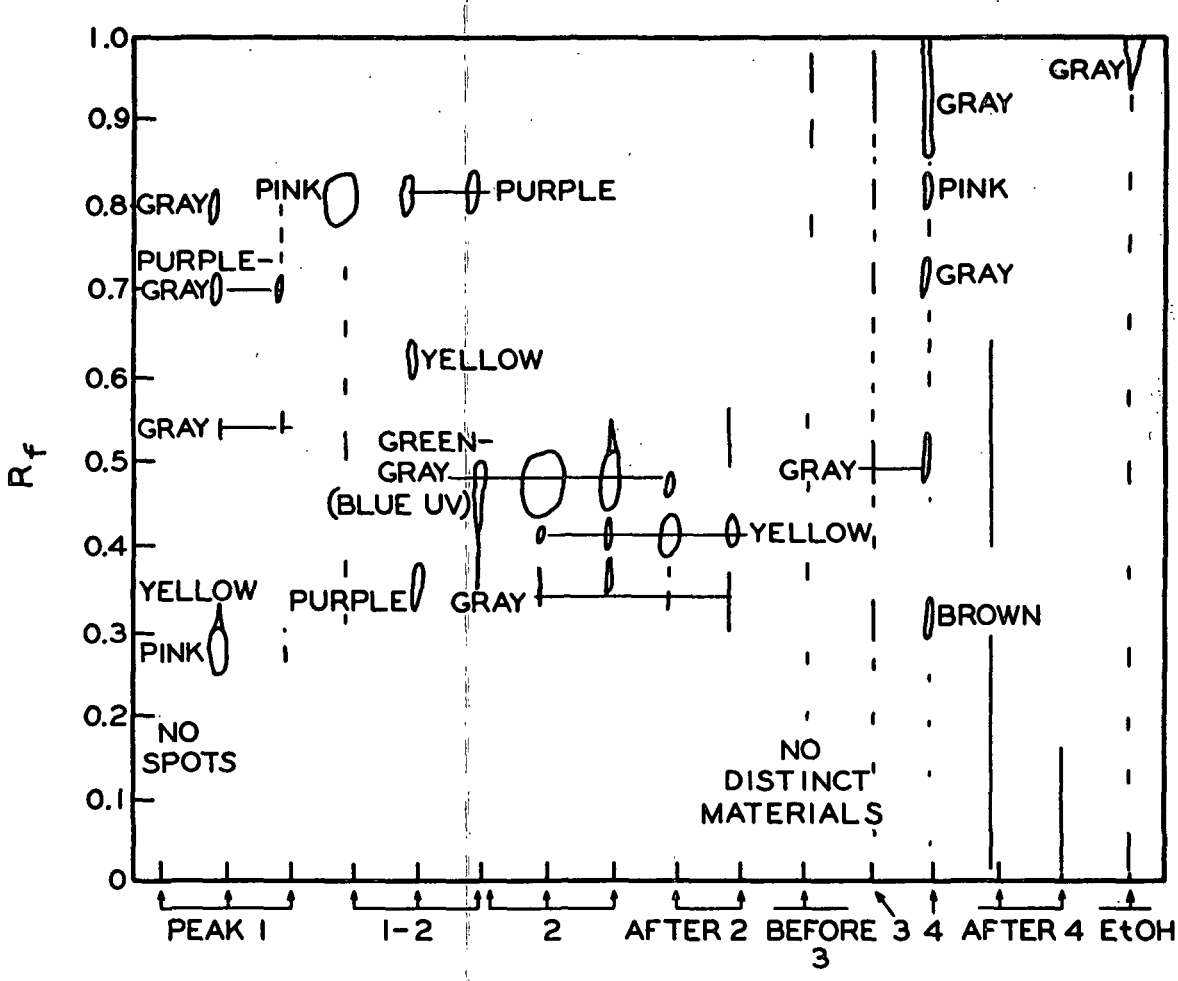

Figure 6. TLC Results from Polyamide Column Chromatography of Ethyl Ether Extract

TABLE III

SUMMARY OF POLYAMIDE COLUMN CHROMATOGRAPHY OF ETHYL ETHER EXTRACT[a]

H_2O Eluted, ml.	Weight,[b] mg.	%	Peak[c]	Materials Detected[c]
400	80	2	1	Trace of Salicin, TLC 0.70 gray
200	150	4	1-2	Salicyl Alcohol, Pyrocatechol
500	1600	39	2	Trichocarpin (687)[d]
600	300	7	After 2	Salireposide (96)
800	150	4	Before 3	Gentisic Acid and derivatives
1200	250	6	3	p-Coumaric Acid (137)
3000	400	9	4	Trichocarposide (175)
17000	210	5	After 4	No distinct materials
Ethanol extraction	980	24		Quercetin (?) & unknown materials
Total	4120			

[a] 4.62 g. put on 2.5 x 36 cm. column.

[b] Material removed from column.

[c] See Fig. 4, 5, and 6.

[d] Values in parentheses give mg. of crystals isolated.

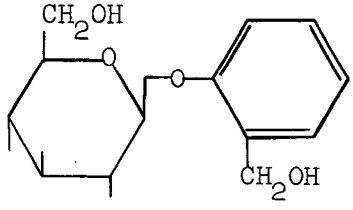

Figure 7. Salicin

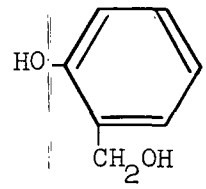

Figure 8. Salicyl Alcohol

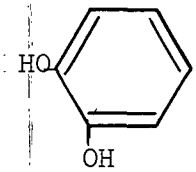

Figure 9. Pyrocatechol

Crystals obtained from fractions indicated by the second and largest peak were composed mainly of trichocarpin (see Fig. 1 and 4). When recrystallized from water and ethanol, they melted[7] at 134-6°, had an IR spectrum identical with that of the authentic trichocarpin[8], and did not depress a mixed melting point with authentic material. Crystals of salireposide (Fig. 10) also appeared with trichocarpin. TLC showed that salireposide appeared in fractions about half way through the second peak and continued until the third. Point 2A on Fig. 4 shows where the greatest amount of crystalline salireposide was obtained. Salireposide was identified by a mixed melting point with authentic material[9]. The weight of materials shown in the second peak of Fig. 4 indicate that over 40% of the ethyl ether extract is trichocarpin and salireposide.

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[7]All melting points were determined with a Thomas-Hoover melting point apparatus.

[8]Obtained from Dr. V. Loeschcke.

[9]Obtained from Dr. Irwin A. Pearl.

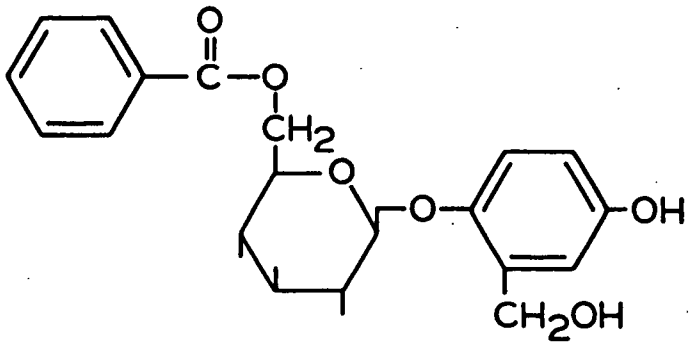

Figure 10. Salireposide

Several materials with ultraviolet fluorescence were detected in fractions immediately before the third peak. One of these was identical to gentisic acid with respect to TLC and paper chromatograms developed in water, sprayed with DPNA and silver, and observed for UV. Some very weak pink spots, which were similar to p-hydroxybenzoic acid, were detected on paper chromatograms of these fractions. A mixture of salireposide and trichocarpin was given a mild acid hydrolysis. The TLC and paper chromatographic results of the hydrolyzate were very similar to those obtained from fractions immediately before the third peak.

Fractions represented by the third peak were composed of p-coumaric acid (Fig. 11). These fractions gave spots identical to known trans p-coumaric acid on paper chromatograms developed in water, BPW, BA, and BFW and sprayed with DPNA. Paper chromatographed trans p-coumaric acid has distinctive colors with UV and DPNA (see Table II). An IR spectrum of this p-coumaric acid had identical peaks with that of known p-coumaric acid. After several recrystallizations from water, the unknown p-coumaric acid melted at 206-7° and then decomposed.

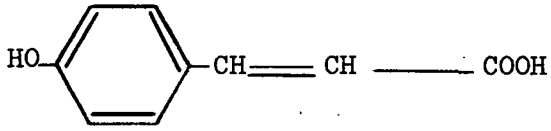

Figure 11. p-Coumaric Acid

Fractions shown by the last peak, Number 4, contained a material which crystallized in the circulating evaporator. A study of the IR spectrum (Fig. 12) of this material, m.p. 180-2°, indicated that it was not a known glycoside. A structural analysis of this new material, trichocarposide, is presented in the next section of this dissertation. Trichocarposide crystals accounted for more than 3.8% of the ethyl ether extract and the peak area in Fig. 5 indicates that it may account for more than 8% of this extract. TLC of trichocarposide gave a weak pink-gray spot at $\underline{R}_f$ 0.45 (the same $\underline{R}_f$ as trichocarpin) with no UV fluorescence.

After Peak 4, another 9.7% of the weight of the extract was removed by elution with 10 more liters of water. These materials showed no distinct TLC or paper chromatographic results. When the polyamide was removed from the column and extracted with ethanol, another 21.2% of the extract was obtained. This material failed to give any distinct TLC results. Paper chromatograms developed in water showed a DPNA and UV yellow spot at $\underline{R}_f$ 0.00 which was similar to quercetin. Since the composition of most of these materials was not known and appeared to be complex, further investigations were not made. Of the original ethyl ether extract, 10.8% was not removed from the column. Since this figure was arrived at by difference, it is subject to the greatest error. Some materials were retained by the column as it darkened after use.

The ethyl ether extract from Bark 1 was chromatographed on a polyamide column. This extract had been unsuccessfully chromatographed on several MN polyamide columns and collected. The fractions from these columns were combined and placed on the same polyamide column used for the ethyl ether extract of Bark 2. Aqueous elution of this column gave fractions from which crystalline trichocarpin, salireposide, and trichocarposide were obtained.

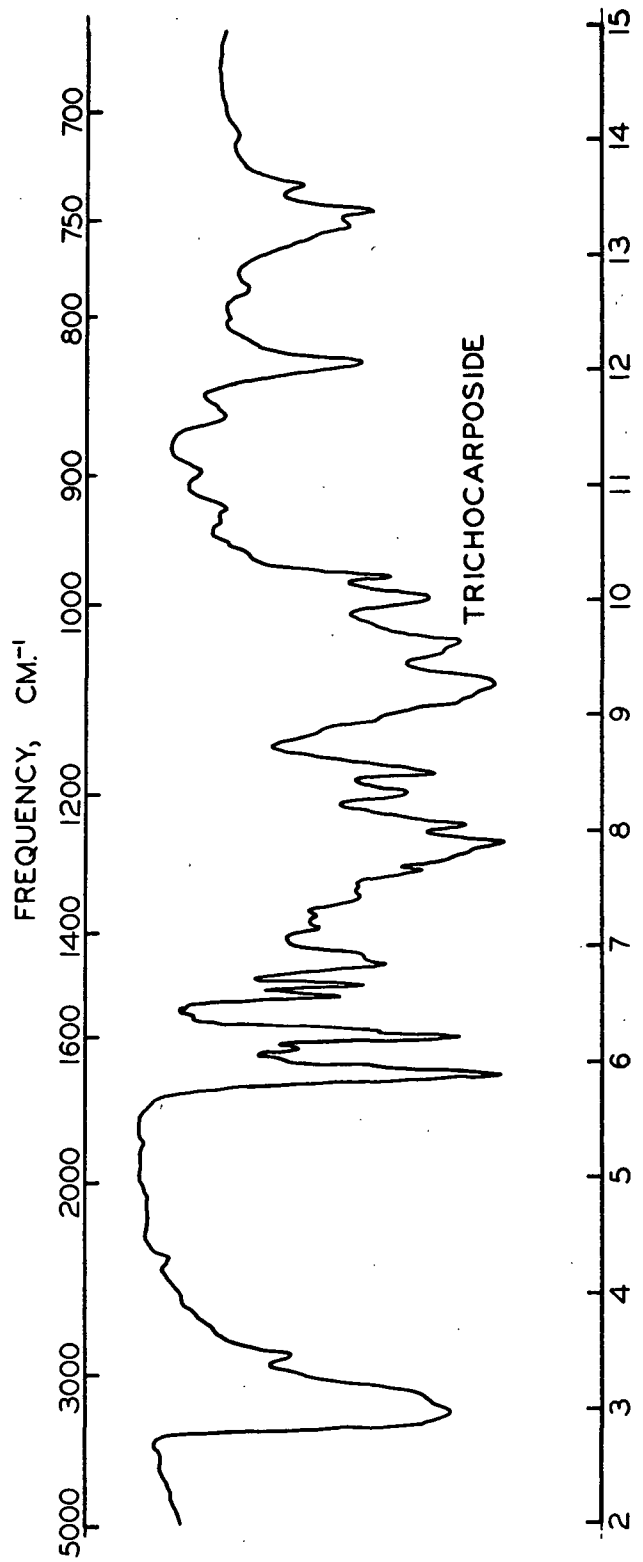

Figure 12. IR Spectrum of Trichocarposide

LEAD SUBACETATE TREATMENT

Part of the ethyl ether extract from Bark 2, 9.57 g., was treated with lead subacetate (see Fig. 3). The ethyl ether extract and 25 g. of lead subacetate were added to 2 liters of water. The mustard-yellow solution was stirred at room temperature (28°) for one hour and filtered through Celite. Both the filtrate and the precipitate were saturated with hydrogen sulfide, boiled, and filtered through Celite in the usual manner (1-8). The material recovered from the filtrate gave 1.18 g. (12%) and the material recovered from the precipitate gave 6.27 g. (66%). Both materials were chromatographed on separate polyamide columns. These chromatograms, which are summarized in Tables IV and V, gave results very similar to those shown in Table III.

SUMMARY

Trichocarpin was the major constituent of the ethyl ether extract. Significant amounts of salireposide, a new glycoside – trichocarposide, and p-coumaric acid and small amounts of pyrocatechol, salicyl alcohol, and salicin also were present. These materials were separated by polyamide column chromatography.

The lead subacetate treatment does not cause a significant change in the ethyl ether extractives. Most of the trichocarpin, salireposide, trichocarposide, p-coumaric acid, and pyrocatechol was found in the materials recovered from the lead precipitate rather than from the lead filtrate. The semiquantitative results indicate that the amount of material present after the mild lead subacetate treatment was the same as before the treatment.

TABLE IV

SUMMARY OF POLYAMIDE COLUMN CHROMATOGRAPHY OF MATERIAL RECOVERED FROM THE LEAD FILTRATE OF THE ETHYL ETHER EXTRACT[a]

H_2O Eluted, ml.	Weight,[b] mg.	%	Peak	Materials Detected
200	180	16	1	Trace of salicin & salicyl alcohol TLC 0.18 yellow, & 0.70 purple
150	62	6	2	Salicyl alcohol TLC 0.60 yellow
400	171	16	3	Pyrocatechol, other black residues
800	396	36	4	Trichocarpin (243)[c] and salireposide (58)
2500	60	5	5?	Trichocarposide (?) & p-coumaric acid (?)
11000	100	9	After 5	No distinct materials
Ethanol extraction	137	12		No distinct materials
Total	1106			

[a] 1.19 g. put on 2.6 x 37 cm. column.

[b] Material removed from column.

[c] Values in parentheses give mg. of crystals isolated.

TABLE V

SUMMARY OF POLYAMIDE COLUMN CHROMATOGRAPHY OF MATERIAL RECOVERED FROM THE LEAD PRECIPITATE OF THE ETHYL ETHER EXTRACT[a]

H_2O Eluted, ml.	Weight[b] mg.	%	Peak	Materials Detected
450	76	2	1	Salicin, salicyl alcohol
400	526	11	1	Pyrocatechol, other black residues
1500	2630	57	2	Trichocarpin (663)[c] & salireposide (107)
700	192	4	After 2	No distinct materials
1500	344	7	3	p-Coumaric acid (106)
3500	585	13	4	Trichocarposide (190)
15000	280	6	After 4	No distinct materials
Total	4633			

[a] 6.27 g. put on 2.6 x 49 cm. column.

[b] Material removed from column.

[c] Values in parentheses give mg. of crystals isolated.

THE STRUCTURE OF TRICHOCARPOSIDE

p-COUMARIC ACID AND SALICIN

Trichocarposide, the material of unknown structure which was isolated from the ethyl ether extract, was found to be p-coumaroyl salicin. This structure was arrived at in the following manner. A study of the IR spectrum of trichocarposide, shown in Fig. 11, suggested an ester. Therefore, a sample was saponified by boiling 20 mg. in a mixture of a 0.4-ml. saturated barium hydroxide solution and 0.1 ml. of water. After cooling, the mixture was acidified with dilute sulfuric acid and the precipitated barium sulfate was removed by filtration. The filtrate was extracted with ethyl ether. This ethyl ether extract gave over 6 mg. of crystalline p-coumaric acid, whose melting point, 218-19°, was not depressed with known p-coumaric acid. The sulfuric acid was removed from the raffinate by adding barium carbonate and filtering. Upon evaporation to dryness, crystalline salicin was obtained. Its melting point, 200-1°, was not depressed with known salicin.

In a similar hydrolysis of another sample, only salicin and cis and trans p-coumaric acid were detected by extensive TLC and paper chromatography using BFW, BPW, BA, and water developers sprayed with DPNA, silver, and indicator. These materials were not detected in the unhydrolyzed trichocarposide.

CARBON AND HYDROGEN

A carbon and hydrogen analysis[10] supports a one to one ratio of p-coumaric acid to salicin. A sample of trichocarposide was recrystallized from water and then dried in an Abderhalden drying apparatus under vacuum at 100° for over eight hours.

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[1] Made by Geller Laboratories, Charleston, West Virginia.

Calculated for $C_{22}H_{24}O_9$: C, 61.10; H, 5.59. Found: C, 61.0; H, 5.6.

WATER OF CRYSTALLIZATION

When part of the trichocarposide was dried in the Abderhalden drying apparatus, 4.7% of its weight was lost. The monohydrate of trichocarposide should lose 4.0% of its weight upon drying. A carbon and hydrogen analysis was made on an air-dried sample of trichocarposide.

Calculated for $C_{22}H_{24}O_9 \cdot H_2O$: C, 58.66; H, 5.82. Found: C, 58.2; H, 5.6.

These results suggest that trichocarposide, recrystallized from water, contains one mole of water of crystallization.

OPTICAL ROTATION

The optical rotation was determined on several samples dissolved in 80% acetone. The rotation was $[\alpha]_D^{20}$ - 11° ($\underline{c}$ = 2.3).

LOCATION OF THE p-COUMAROYL GROUP

Trichocarposide is a p-coumaroyl salicin. From the structure of salicin, it is evident that there are five possible locations for the p-coumaroyl group. These are shown in Fig. 13. Because of the small amount of material, a methylation was not feasible to show the location of the p-coumaroyl group. Instead, several indirect methods were used to suggest the location of the p-coumaroyl group.

PERIODATE OXIDATION

Evidence about the position of the p-coumaroyl group was obtained from a study of the periodate oxidation of trichocarposide. A scaled-down version of the methods (23-25) used on tremuloidin (3, 26) was developed to determine the moles of periodate consumed. Ten milligrams of glucoside were weighed into a

15-ml. black vial, 5 ml. of methanol were added, the solution was cooled, and then 10 ml. of 0.02M sodium periodate were added. The vials were capped, shaken, and placed in a water bath at 5°. At appropriate times duplicate 1-ml. samples were removed and an excess of standard sodium arsenite was added. After 15 minutes, the samples were titrated with iodine. This titration was made at 5° to avoid the reaction of iodine with p-coumaric acid. Grandidentatin (a 2-p-coumaroyl glucoside), salicin, and a blank were run as controls.

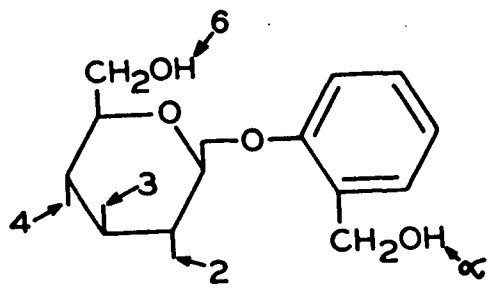

Figure 13. Possible Locations of p-Coumaroyl Group on Salicin

The results of one run are presented in Table VI and are plotted in Fig. 14. Salicin consumed almost two moles of periodate with no significant overoxidation. Grandidentatin appeared to consume one mole of periodate and trichocarposide appeared to consume two; however, both showed significant amounts of overoxidation. During this and several other similar runs, it was observed that trichocarposide always consumed about one more mole of periodate than grandidentatin. This suggests that the p-coumaroyl group is located at the alpha or six position (see Fig. 13).

Several attempts were made to isolate significant products of the periodate oxidation. Formic acid is formed if there is no substitution at the two, three, or four position. Attempts to titrate the formic acid with iodine (25, 26) were

not successful with the very small samples. With larger samples, significant amounts of acid were detected, as shown in Table VI. This further substantiated the theory that the p-coumaroyl group was located at the alpha or six position.

TABLE VI

PERIODATE OXIDATION DATA

Sample	8 Hr.	22 Hr.	50 Hr.	75 Hr.	Theory	Acids at 75 Hr.
Trichocarposide	0.6[a]	2.0	2.6	3.4	–	1.1[b]
Grandidentatin	–	1.0	1.8	2.6	1.0	0.4
Salicin	0.3	1.9	1.9	2.0	2.0	0.8

[a]Moles of oxidant consumed per mole of glycoside after the given number of hours.
[b]Moles of acid liberated per mole of glycoside.

ENZYMATIC HYDROLYSES

Enzymatic hydrolyses with beta glucosidase were used in an attempt to determine if the p-coumaroyl group was on the alpha position. In the first attempted hydrolysis, 47 mg. of trichocarposide were added to 10 ml. of 0.01M acetate buffer (pH 5.4). The glucoside was dissolved by heating but recrystallized upon cooling. About 10 mg. of beta glucosidase[11] were added to the solution. After 18 hours and again after 42 hours of standing at 37°, one third of the material was removed. The solutions were boiled to dissolve the crystals and inactivate the enzyme, and filtered. The filtrate was concentrated with vacuum evaporation and spotted on thin-layer and paper chromatograms. Trichocarpin was run as a control. TLC and paper chromatography showed that glucose was present in the trichocarpin hydrolyzate, but not in the trichocarposide hydrolyzate.

[11]Obtained from Nutritronal Biochemicals Corporation, Cleveland, Ohio.

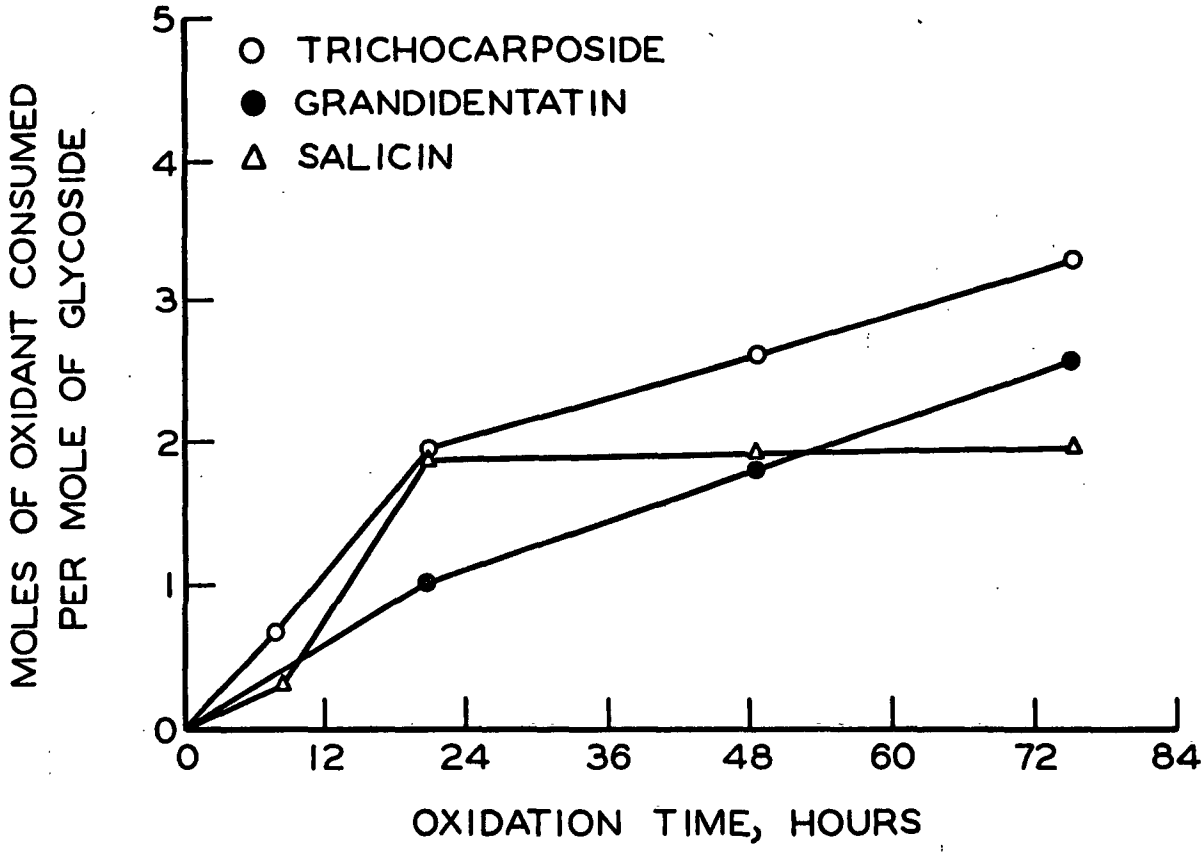

Figure 14. Periodate Consumption

In another enzymatic hydrolysis, 20 mg. of trichocarposide were added to 1 ml. of water at 80°. Trichocarposide was insoluble at this concentration. Five milliliters of 0.01M acetate buffer (pH 5.07) were added and the solution was heated to 80°. Grandidentatin and trichocarpin were given similar treatments. The trichocarpin was soluble in the solution, whereas the grandidentatin was not. About 10 mg. of beta glucosidase were added to each of the samples. After several hours at 80°, the temperature was gradually lowered to 60° with periodic additions of beta glucosidase. Finally, after 24 hours, portions of the hydrolyzate were spotted on paper and thin-layer chromatograms. TLC showed little evidence of reaction. Paper chromatograms, developed in EPW and dipped in silver, showed only a weak glucose spot for the trichocarpin hydrolyzate. Paper chromatograms, developed in water and inspected for UV and then sprayed with DPNA and silver, mainly gave spots of the starting materials. The hydrolyzates of grandidentatin and trichocarposide also showed weak spots at R_f 0.65. These spots were a weak purple blue with DPNA, showed a weak UV fluorescence, and gave a weak spot with silver. Possibly, these spots were from p-coumaroyl esters of glucose since they had characteristics of both glucose and p-coumaric acid. Other p-coumaroyl esters of glucose, which have similar chromatographic results, have been reported (27-30). In addition, the trichocarpin hydrolyzate also gave weak spots of glucose and trichocarpigenin (trichocarpin aglucone).

In an attempt to obtain more hydrolyzate, the temperature of the solutions was maintained at 40-60° for two days. Periodically, more beta glucosidase was added. TLC and paper chromatography showed a strong spot of glucose from the trichocarpin hydrolyzate. The grandidentatin hydrolyzate gave a possible trace of glucose and p-coumaric acid. The trichocarposide hydrolyzate gave no detectable glucose.

These enzyme results support the premise that the p-coumaroyl group is attached to the glucose rather than the salicyl alcohol part of the molecule. If the converse were true, a trace of glucose would be observed with any hydrolysis – none was observed. Furthermore, a trace of p-coumaroyl glucose may have been detected in the trichocarposide and grandidentatin hydrolyzates.

Thieme (9) observed that salicin, picein, and triandrin were hydrolyzed by emulsin (crude beta glucosidase), while populin, tremuloidin, fragilin, salireposide, and grandidentatin were not. Thus, it appears that simple glucosides are hydrolyzed, while those that have an ester group on the glucose ring are not. Trichocarposide apparently has an acyl group on the glucose ring and, consequently, does not react readily with beta glucosidase.

SUMMARY

A mild alkaline hydrolysis of trichocarposide gave only salicin and p-coumaric acid. A carbon and hydrogen analysis indicated that the empirical formula of trichocarposide was $C_{22}H_{24}O_9$. Periodate oxidations and enzymatic hydrolyses of trichocarposide were used to determine the location of the p-coumaroyl group. The periodate oxidation indicated that the p-coumaroyl group was on the alpha or six position of salicin while the enzymatic hydrolysis showed that it was not probable that the p-coumaroyl group was at the alpha position (see Fig. 13). Thus, this evidence shows that trichocarposide is 6-O-p-coumaroyl salicin [o-hydroxymethylphenyl 6-O-(p-hydroxycinnamoyl)-β-D-glucopyranoside]. This structure is shown in Fig. 15.

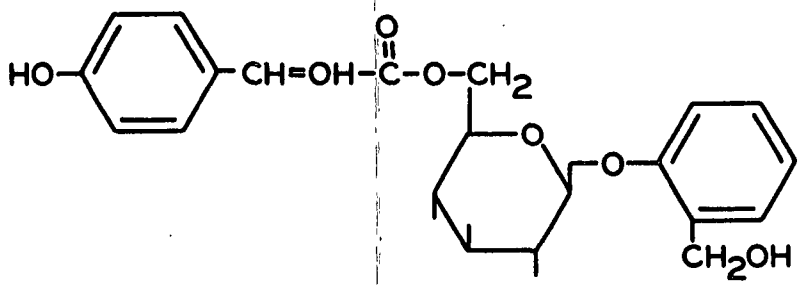

Figure 15. Trichocarposide

CHLOROFORM EXTRACT

INTRODUCTION

The hot water extract was extracted exhaustively with chloroform. The chloroform extract was investigated by direct polyamide column chromatography. Also, part of the extract was treated with lead subacetate, and the resulting deleaded filtrate and reconstituted deleaded precipitate were chromatographed on polyamide columns. Part of the extract was given a mild acid hydrolysis, and the hydrolyzate was chromatographed using a polyamide column. A flow chart of these procedures is shown in Fig. 16.

PRECIPITATE

The chloroform extract was concentrated and dissolved in ethanol and tetrahydrofuran before processing. As the tetrahydrofuran evaporated, a precipitate, which comprised over 10% of the chloroform extract, formed in the solution. As this precipitate appeared to be composed of fatty materials, it was not investigated further.

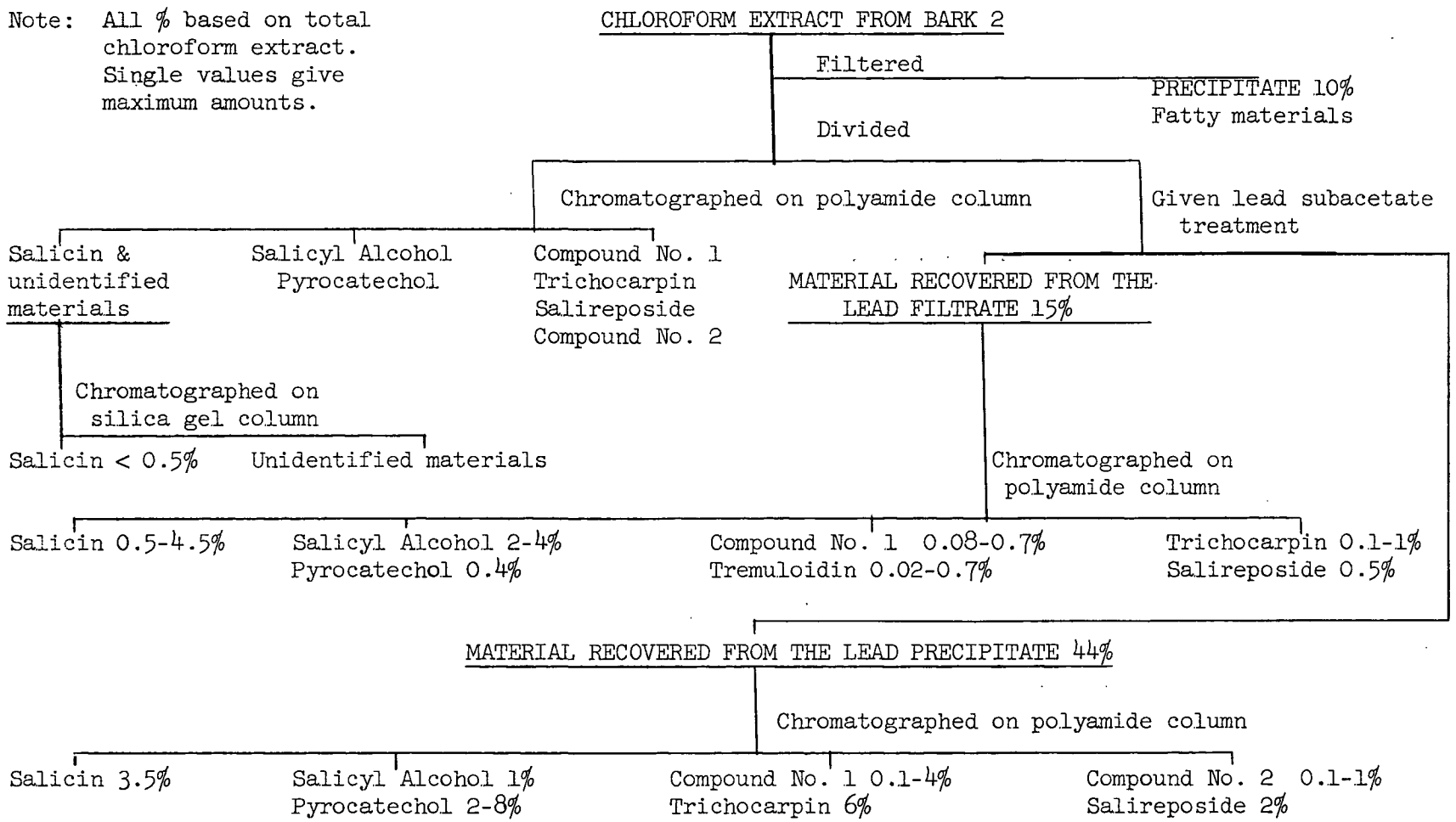

Figure 16. Flow Chart of Processing of Chloroform Extract

LEAD SUBACETATE TREATMENT

A sample, 10.4 g., of chloroform extract was evaporated to dryness and then added to 2000 ml. of water and 27 g. of lead subacetate. This mustard-yellow mixture was stirred at room temperature (28°) for one hour and then filtered. The lead precipitate was suspended in 3 liters of water, saturated with hydrogen sulfide, boiled, filtered, and evaporated to dryness. This procedure yielded 5.10 g. (49%) of material. The lead filtrate was treated in the same manner and yielded 1.72 g. (17%) of dark brown oil.

MATERIAL RECOVERED FROM THE LEAD FILTRATE

The entire 1.72-g. sample of material recovered from the lead filtrate of the chloroform extract was slurried in 50 ml. of water and placed on a polyamide column. The column was eluted with 22 liters of water. Figure 17 shows the elution diagram, Fig. 18, the TLC results, and Table VII, a summary of the chromatography.

The first materials removed from the column contained salicin plus a trace of glucose and several other materials. Over 60 mg. of salicin crystals were obtained.

After salicin, salicyl alcohol was obtained. Salicyl alcohol was initially identified by TLC and paper chromatography with DPNA in water, BPW, and BA. The fractions which contained salicyl alcohol were dissolved in acetone and filtered. The filtrate was evaporated to give a semisolid material (m.p. 65-74°). An IR spectrum of this material had identical peaks with that of known salicyl alcohol.

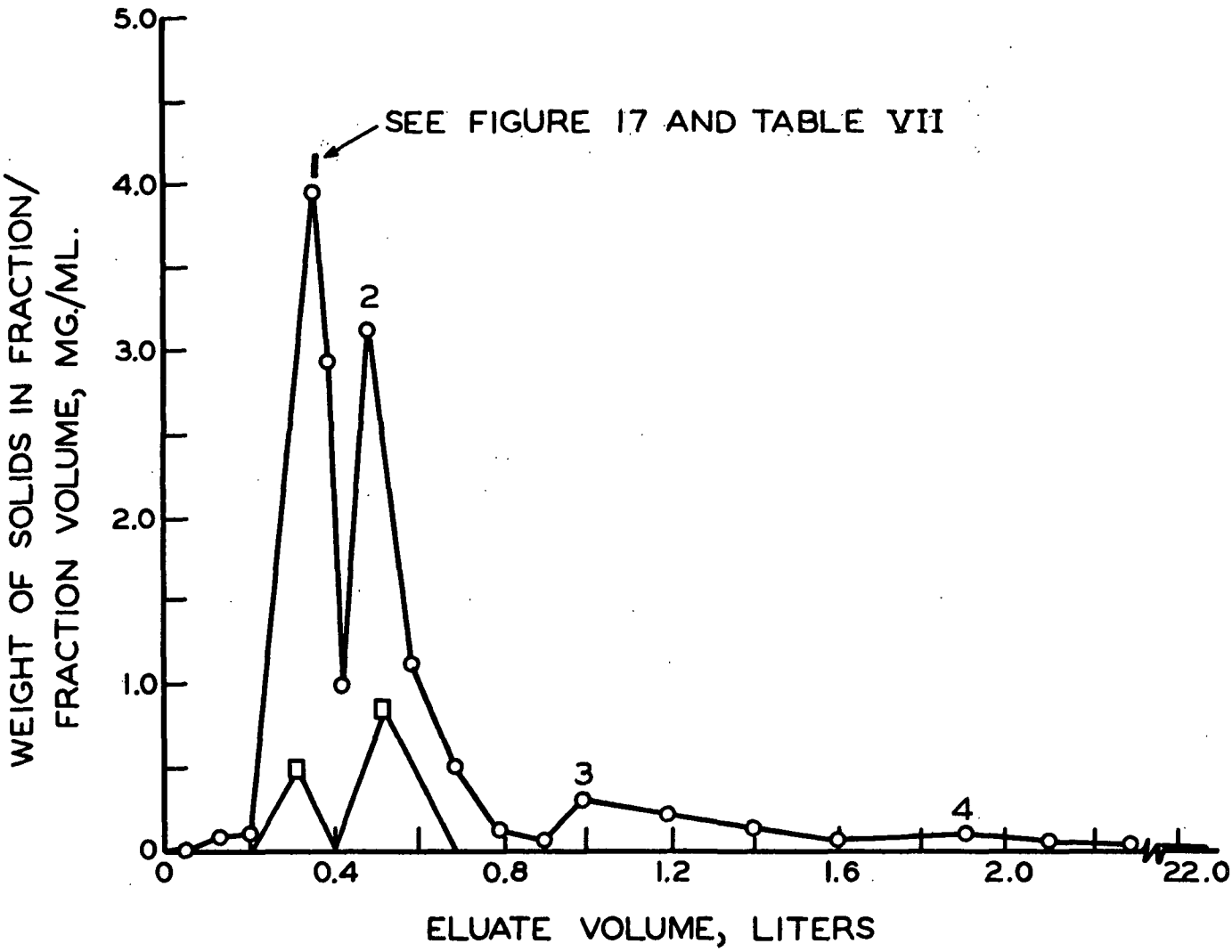

Figure 17. Elution Diagram of Polyamide Column Chromatography of Material Recovered from the Lead Filtrate of the Chloroform Extract

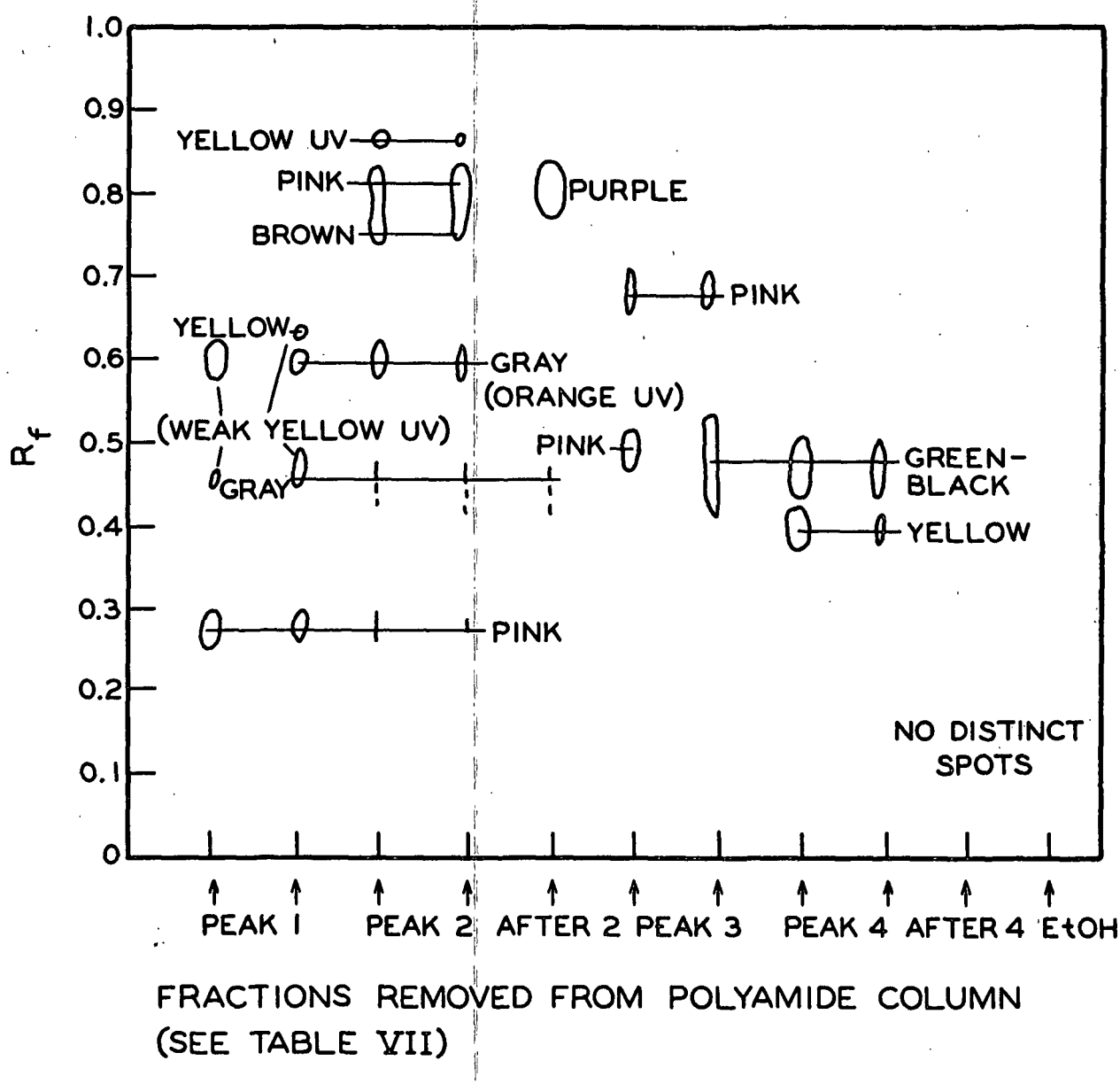

Figure 18. TLC Results of Polyamide Column Chromatography of Material Recovered from the Lead Filtrate of the Chloroform Extract

TABLE VII

SUMMARY OF POLYAMIDE COLUMN CHROMATOGRAPHY OF MATERIAL RECOVERED FROM THE LEAD FILTRATE OF THE CHLOROFORM EXTRACT[a]

H_2O Eluted, ml.	Weight,[b] mg.	%	Peak[c]	Materials Detected[c]
400	473	32	1	Salicin (60)[d]
300	401	27	2	Salicyl Alcohol (200), other black residues
200	43	3	After 2	Pyrocatechol, black residues
600	110	7	3	Compound No. 1 (10), Tremuloidin (3)
2500	127	10	4	Trichocarpin (7), and Salireposide
18000	116	9	After 4	No distinct materials
Ethanol elution	213	14		No distinct materials
Total	1483			

[a] 1.72 g. put on 3.1 x 47 cm. column.

[b] Materials removed from column.

[c] See Fig. 17 and 18.

[d] Values in parentheses give mg. of crystals isolated.

Subsequent fractions contained pyrocatechol which was identified by TLC and paper chromatography with DPNA in water, BPW, and BA.

Trace amounts of two crystalline materials were removed from the column after the pyrocatechol. One material, Compound No. 1, which gave 10 mg. of white crystals, melted at 162-165° and had a TLC spot identical to trichocarpin. Enzymatic hydrolysis, using the same procedures as were used on trichocarposide, gave glucose and a material with a TLC spot identical to trichocarpigenin (trichocarpin aglucone). Further identification was not possible.

The other material consisted of 3 mg. of white crystals which melted at 209-10° and had an IR spectrum identical with that of tremuloidin.

After the tremuloidin, very small amounts of trichocarpin and salireposide were removed from the column. No distinct materials were obtained after the salireposide.

MATERIAL RECOVERED FROM THE LEAD PRECIPITATE

The entire 5.10 g. sample of material recovered from the lead precipitate of the chloroform extract was placed on a polyamide column and eluted with water. A summary of this chromatography is shown in Table VIII.

TABLE VIII

SUMMARY OF POLYAMIDE COLUMN CHROMATOGRAPHY OF MATERIAL[a] RECOVERED FROM THE LEAD PRECIPITATE OF THE CHLOROFORM EXTRACT

H_2O Eluted, ml.	Weight,[b] mg.	%	Peak	Materials Detected
430	282	8	1	Trace of Salicin & Glucose
200	143	4	Small 2	Salicyl Alcohol, TLC 0.33, 0.40, 0.50 gray
450	670	18	3	Pyrocatechol, black materials
450	325	9	3-4	Compound No. 1 (8)[c]
900	792	22	4	Trichocarpin (18), and Salireposide
1500	443	12	Small 5	Compound No. 2 (14), TLC 0.60, 0.70, 0.90 gray
18000	1011	27	After 5	No distinct materials
Total	3666			

[a] 5.10 g. put on 3.4 x 42 cm. column.

[b] Material removed from column.

[c] Values in parentheses give mg. of crystals isolated.

TLC and paper chromatography detected a trace of salicin in the first fractions. Salicyl alcohol was detected in fractions represented by the second peak. At the start of Peak 3, blue solutions containing large amounts of pyrocatechol were removed from the column. These fractions were evaporated to dryness and extracted with low-boiling petroleum ether yielded crystals of pyrocatechol. An IR spectrum of these oily crystals had peaks identical with that of known pyrocatechol.

A trace amount of white crystals was obtained from fractions after the pyrocatechol. The IR spectrum of these crystals, m.p. 163-5°, is shown in Fig. 19. These crystals also gave a TLC spot identical to trichocarpin. Enzymatic hydrolysis gave a trace of glucose. This material appeared to be Compound No. 1. A trace of this material was found when another lead subacetate-treated chloroform extract was chromatographed on a polyamide column.

Small amounts of trichocarpin and salireposide were obtained from fractions after Compound No. 1. In addition, a second unidentified material, m.p. 163-5°, was obtained from these fractions. TLC gave a spot almost identical to trichocarpin. After an enzymatic hydrolysis, glucose was detected. A study of the IR spectrum of this material (Fig. 20) indicated that it was another unidentified material, Compound No. 2. More Compound No. 2 was obtained from another polyamide column chromatogram of material recovered from a leaded chloroform extract.

POLYAMIDE COLUMN CHROMATOGRAPHY

The chloroform extract was chromatographed directly on two polyamide columns. Both gave poor results. TLC and weight results indicated some chromatographic separation. Most of the fractions contained very unstable materials which turned black in a few days. Elution with ethanol gave waxy

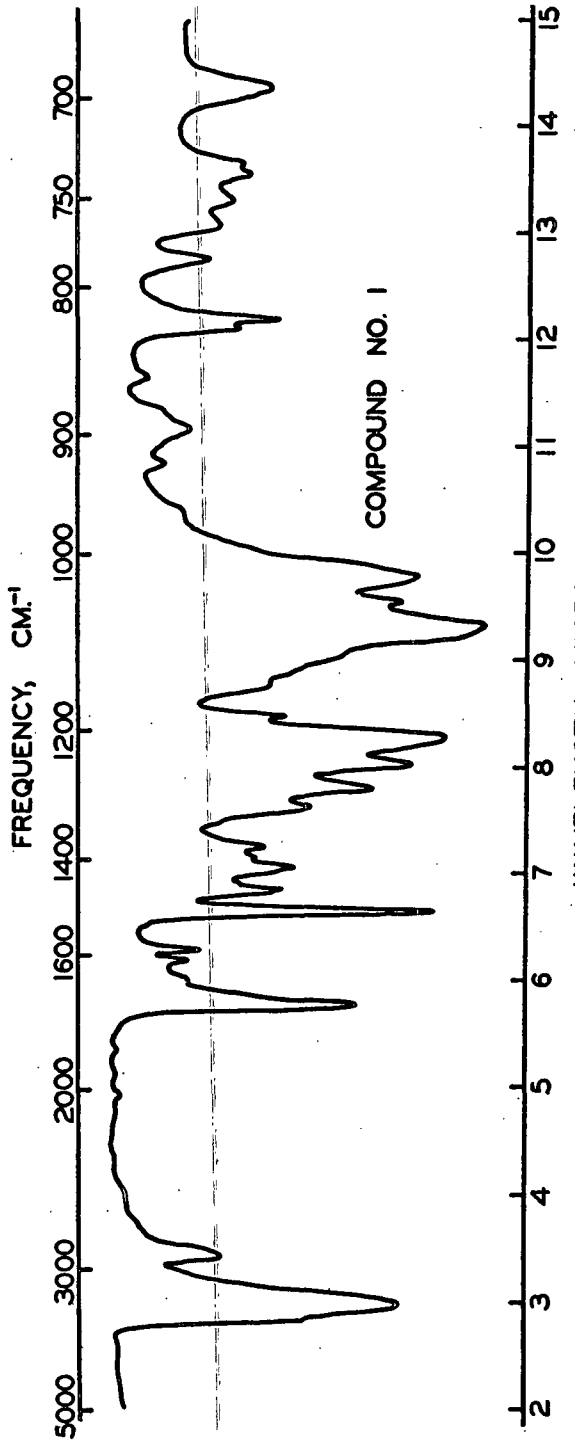

Figure 19. IR Spectrum of Compound No. 1

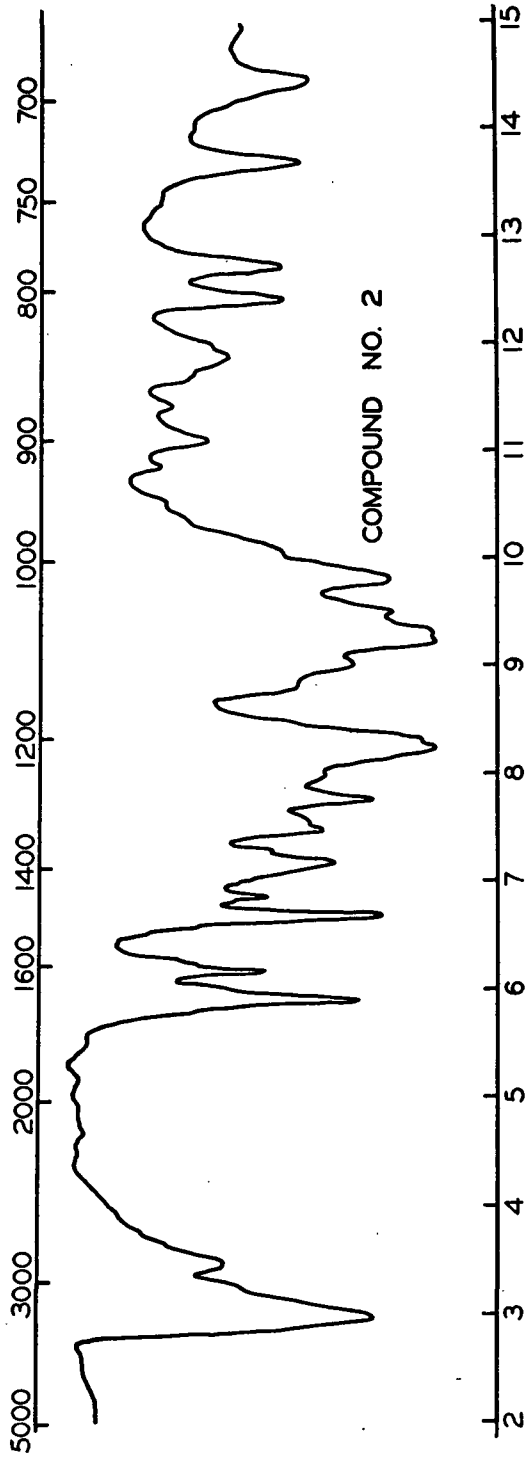

Figure 20. IR Spectrum of Compound No. 2

materials. Very little extract was removed from the column. Trace amounts of some materials were obtained. These chromatograms are summarized in Table IX.

The fractions from the first peak of one column were combined and placed on a 2.0 x 31 cm. column which was dry packed with 45 g. of silica gel 0.05 - 0.20[1]. This column was eluted with 4:1 chloroform-methanol. TLC and weight results showed that a trace of salicin was present. The fractions which contained the trace of salicin represented less than 0.5% of the chloroform extract.

TABLE IX

SUMMARY OF POLYAMIDE COLUMN CHROMATOGRAPHY OF CHLOROFORM EXTRACT

Peak	Materials Detected
1	Trace of Salicin plus six other materials
2	Salicyl Alcohol, Pyrocatechol, Compound No. 1, and five other materials
3	Trichocarpin, Salireposide, plus six other materials
After 3	No distinct materials
Ethanol elution	Waxy materials, no distinct TLC spots
	Large quantity of material not removed from column

[1]Manufactured by E. Merck A. G., Germany.

MILD ACID HYDROLYSIS

Six grams of chloroform extract were subjected to a mild acid hydrolysis by adding seven grams of sulfuric acid in 225 ml. of water and 75 ml. of ethanol to the chloroform extract. The mixture was boiled under reflux for one hour and evaporated under reduced pressure to remove the ethanol (6, 8, 31). This procedure was used for all mild acid hydrolyses in this study. After several days refrigeration, several grams of oily precipitate were obtained. The solution was decanted and concentrated to give more oily precipitate. This oily precipitate was slurried in a small amount of water and placed on a large (3.3 x 87 cm.) polyamide column. After elution with 14 liters of water, only a trace of materials (240 mg.) was removed. These materials were characteristic of other chloroform extracts, although only trichocarpin and salireposide were identified. Salicin, salicyl alcohol, and pyrocatechol did not appear to be present (probably because of their high solubility in the residual aqueous solution). No salicyloyl glucosides were obtained, although TLC did show some pink spots at R_f 0.49 and 0.60. These spots came from fractions which contained 60 mg. of material.

SUMMARY

The chloroform extract contained a large amount of unidentified fatty material. These materials were not removed from an aqueous eluted polyamide column chromatogram. Alcohol elution removed complex waxy materials with no significant chromatography spots.

Salicyl alcohol and pyrocatechol were present in the chloroform extract. An accurate determination of the amount of these materials could not be obtained because chromatographic separation was poor. After a mild lead subacetate treatment, salicyl alcohol was present in the lead filtrate and pyrocatechol, in the reconstituted precipitate.

Small amounts of salireposide, trichocarpin, and two unidentified materials were isolated. The presence of salireposide and trichocarposide indicated their slight solubility in the exhaustive chloroform extract. The two unidentified materials were both similar to trichocarpin. Trace amounts of both unidentified materials were obtained from several polyamide column chromatograms.

A significant amount of salicin was detected after the mild lead subacetate treatment, whereas before the treatment, only a trace (< 0.5%) was detected. This indicates that some material was hydrolyzed by the lead treatment to give salicin. A trace amount of tremuloidin was found in the lead filtrate. The mild acid hydrolysis did not produce any new materials.

ETHYL ACETATE EXTRACT

INTRODUCTION

Part of the ethyl acetate extract was chromatographed on several polyamide columns both before and after treatment with lead subacetate and a mild acid hydrolysis. A summary of these procedures is shown in Fig. 21 and 22.

POLYAMIDE COLUMN CHROMATOGRAPHY

The ethyl acetate extract was chromatographed on several polyamide columns. For one chromatogram, 22.5 g. of ethyl acetate extract were placed on a 3.5 x 86 cm. column and eluted with 24 liters of water. This chromatogram is summarized in Table X. The first fractions removed from the column contained salicin, glucose, fructose, sucrose, and several other materials. The salicin, which was identified by TLC, was obtained by evaporating these fractions to dryness and dissolving the residue in acetone to leave the insoluble salicin. Only 300 mg. (1.3%) of acetone insoluble (crude salicin) material were obtained. Paper

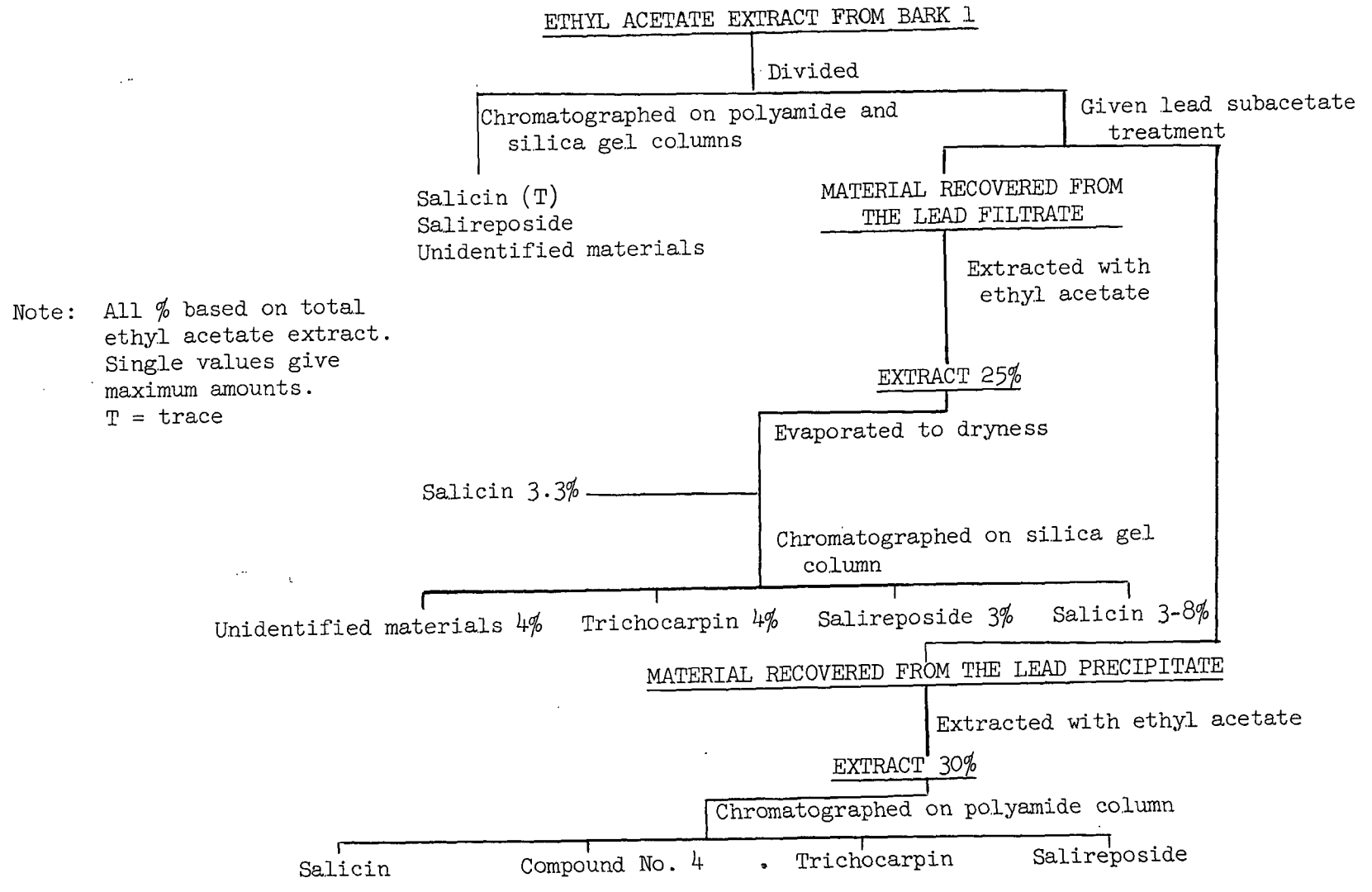

Figure 21. Flow Chart of Processing of Ethyl Acetate Extract

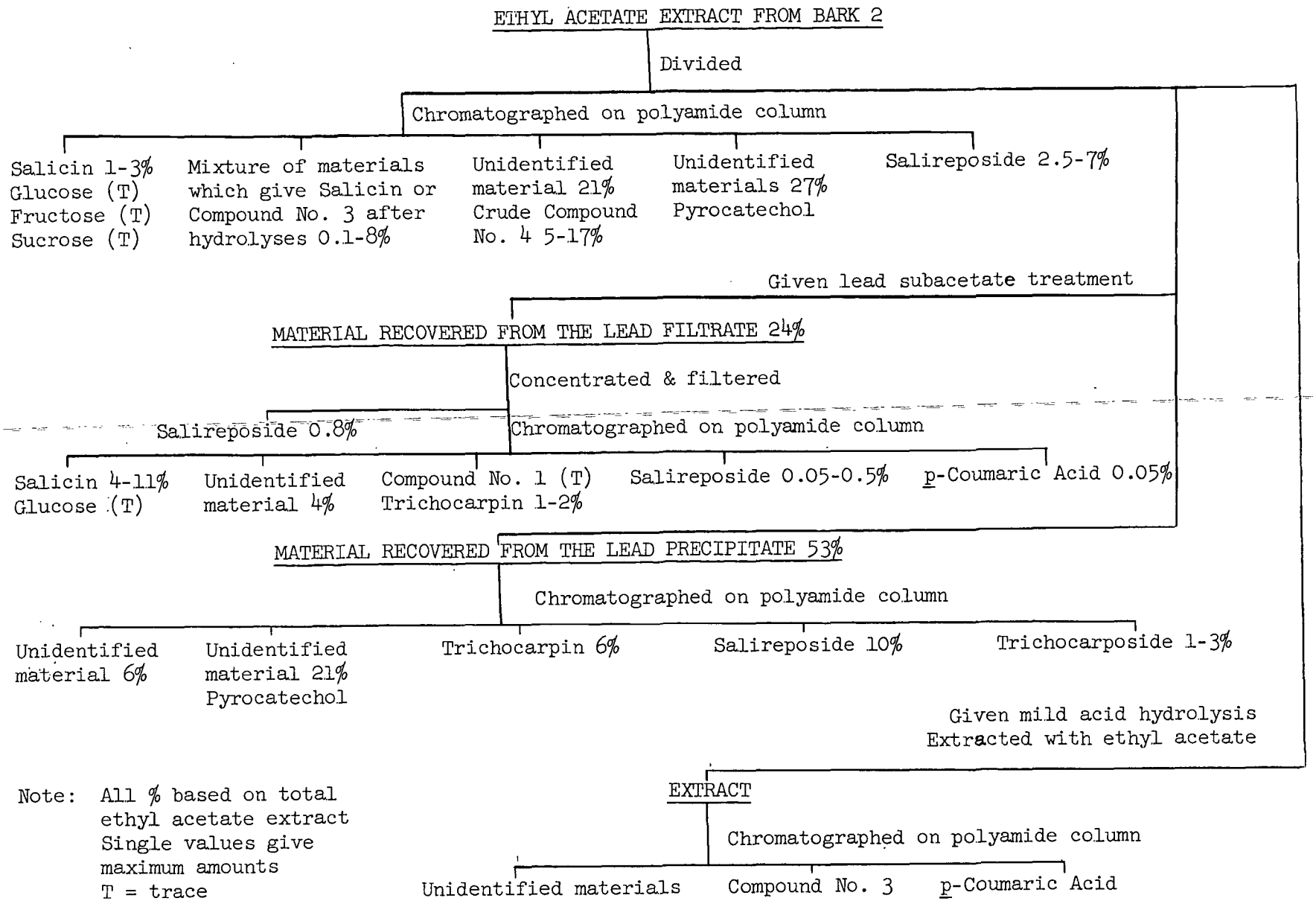

Figure 22. Flow Chart of Processing of Ethyl Acetate Extract

TABLE X

SUMMARY OF POLYAMIDE COLUMN CHROMATOGRAPHY OF ETHYL ACETATE EXTRACT[a]

H_2O Eluted, ml.	Weight,[b] mg.	%	Peak	Materials Detected
400	1500	9	1	Salicin (300)[c], (Compound No. 3)
700	3650	21	2	Unidentified yellow white solid
950	2950	17	After 2	Crude Compound No. 4 (1200)
2400	4610	27	3	Unidentified mixture, Pyrocatechol
3700	1200	7	After 3	Salireposide (480)
15000	880	5	After 3	No distinct materials
Ethanol extraction	2300	14		No distinct materials
Total	17090			

[a] 22.5 g. put on 3.5 x 86 cm. column.

[b] Material removed from column.

[c] Values in parentheses give mg. of crystals isolated.

chromatograms, developed in EPW and treated with silver and anisidine sprays, detected a trace amount of glucose, while urea spray detected trace amounts of fructose and sucrose.

Extensive TLC and paper chromatography did not detect many other significant materials. TLC showed a series of pink-gray spots from R_f 0-0.6 (especially 0.4-0.5) and a yellow spot at R_f 0.18. DPNA sprayed paper chromatograms gave very strong pink spots at R_f 0.85 in water, 0.35 and 0.43 in BA, and 0.65 in BPW. When similar fractions from another column were treated with lead subacetate, more salicin was obtained and when given a mild acid

hydrolysis, an unidentified material, m.p. 170-3°, was obtained. This material, Compound No. 3, gave a TLC and paper chromatography spot similar to salicyloyl salicin. All 7 mg. of this material were hydrolyzed with boiling barium hydroxide, acidified with sulfuric acid, and extracted with ethyl ether. The evaporated ethyl ether extract gave long needle crystals which melted at 156-7°. The melting point and paper chromatography indicated that these crystals were salicylic acid. TLC and paper chromatography of the ether raffinate detected only salicin. Thus, the first peak contains an unidentified mixture of materials which gave salicin or Compound No. 3 after hydrolysis.

Later fractions (Peak 2 of Table X) gave complex paper chromatogram spots that were yellow and blue with DPNA and had strong UV fluorescence. Their R_fs were 0.5-0.8 in water, 0-0.35 in BA, and 0.4-0.9 in BPW. These fractions were evaporated to dryness to give a sirup which was completely soluble in acetone. When evaporated to dryness and extracted with benzene and then ethyl acetate, 800 mg. of white-yellow solid material remained. This solid material, m.p. 150-170°, was shown by TLC and paper chromatography to be a mixture of several similar materials. Further investigations were not made on this material.

Over 1200 mg. of very oily crystalline material were obtained upon evaporation of fractions after the second peak (see Table X). Extraction with acetone left a white material which melted at 170-190° and had a weak pink-gray TLC spot at R_f 0.35. The IR spectrum of this crude material is shown in Fig. 23. A study of this spectrum shows the possibility of a p-coumaroyl compound, Compound No. 4. When part of this material was hydrolyzed with boiling barium hydroxide and acidified with sulfuric acid, large amounts of p-coumaric acid were detected. Enzymatic hydrolysis with beta glucosidase did not give a significant reaction. This material was an unidentified p-coumaroyl compound,

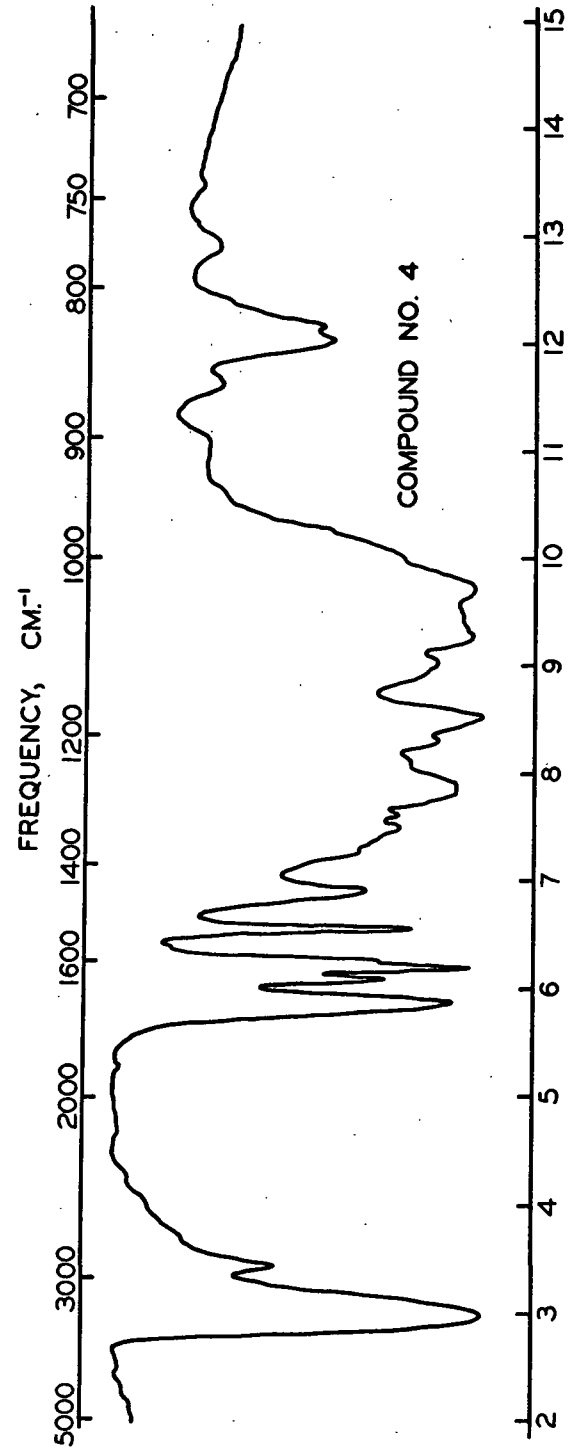

Figure 23. IR Spectrum of Compound No. 4

possibly another p-coumaroyl ester of a glucoside. Further investigations were not made on this material.

Fractions indicated by the third peak were a mixture of several unidentified materials. TLC and paper chromatography detected a weak pyrocatechol spot and several other materials. Unlike the other fractions containing pyrocatechol, these yellow brown oils never blackened.

After the third peak, crystals of salireposide were obtained. Crystalline salireposide accounted for less than 3% of the material removed from the column. No detectable materials were obtained after the salireposide. The top portion of the polyamide column was removed and extracted with ethanol to give 1.46 g. of material. Later, the rest of the polyamide was given a similar extraction yielding 0.84 g. of material. No distinct materials were detected in either extract.

LEAD SUBACETATE TREATMENT

Part of the ethyl acetate extract from each bark was treated with lead subacetate (see Fig. 21 and 22). For Bark 2, 18.3 g. of extract and 51 g. of lead subacetate were placed in 2500 ml. of water. This mustard-yellow solution was stirred at room temperature (24°) for one hour and then filtered. Both the filtrate and the precipitate were saturated with hydrogen sulfide, boiled, and filtered through Celite. The material recovered from the filtrate contained 4.35 g. (24%) and the material recovered from the precipitate, 9.70 g. (53%). Similar results were obtained from the lead subacetate treatment of Bark 1.

MATERIAL RECOVERED FROM THE LEAD FILTRATE

The material recovered from the lead filtrate of the ethyl acetate extract, 4.35 g., was concentrated to a small volume and allowed to stand at 5°.

After several days, 145 mg. of salireposide crystals were obtained. These were identified by TLC and confirmed by a mixed melting point.

The remaining filtrate, 4.20 g., was placed on a 2.5 x 37 cm. polyamide column and eluted with water. This chromatogram is summarized in Table XI. Three weight peaks were obtained from the chromatogram. Fractions shown by the first peak contained large amounts of salicin. Over 700 mg. of crude crystalline salicin were obtained. This represents more than 4% of the original ethyl acetate extract.

Fractions shown by the next peak contained several materials which could not be identified. These fractions were combined and given a mild acid hydrolysis. TLC and paper chromatography did not detect any new materials in the hydrolyzate.

Fractions represented by the third peak were composed of trichocarpin and a trace of salireposide. Most of the salireposide was removed as crystals before putting this extract on the polyamide column. As with other polyamide column chromatograms, the fractions immediately before the trichocarpin contained a very small amount (3 mg.) of unidentified material which could be Compound No. 1.

The column was eluted with 20 more liters of water. Paper chromatograms sprayed with DPNA detected p-coumaric acid, but no other distinct materials.

In the lead subacetate treatment of Bark 1, the material recovered from the lead filtrate was extracted exhaustively with ethyl acetate yielding 3.0 g. (25%) of extract. When this extract was evaporated to dryness and tetrahydrofuran was added, crystals of salicin were obtained. This material was recrystallized from methanol to give over 400 mg. of salicin.

TABLE XI

SUMMARY OF POLYAMIDE COLUMN CHROMATOGRAPHY OF MATERIAL RECOVERED FROM THE LEAD FILTRATE OF THE ETHYL ACETATE EXTRACT[a]

H_2O Eluted, ml.	Weight,[b] mg.	%	Peak	Materials Detected
400	2000	48	1	Salicin (716)[c], Trace of Glucose TLC 0.18 yellow
300	750	18	2	TLC 0.28, 0.38, 0.73 grays and purples (After mild acid hydrolysis, no new materials)
800	450	11	3	Trichocarpin (68), Salireposide (9)
20000	200	5	After 3	Trace of p-Coumaric Acid, no other distinct materials
Total	3400			

[a] 4.20 g. put on 2.5 x 37 cm. column.

[b] Material removed from column.

[c] Values in parentheses give mg. of crystals isolated.

The remaining extract was placed on a 3.5 x 32 cm., 5:1 silica gel-Celite column and eluted with 4:1 chloroform-methanol. The TLC results of fractions obtained from this column are shown in Fig. 24 and a summary is presented in Table XII.

The TLC results (Fig. 24) show that some separation was obtained. Crystals of salireposide and salicin were obtained from the column. TLC results were similar to those obtained from polyamide column chromatography of Bark 2.

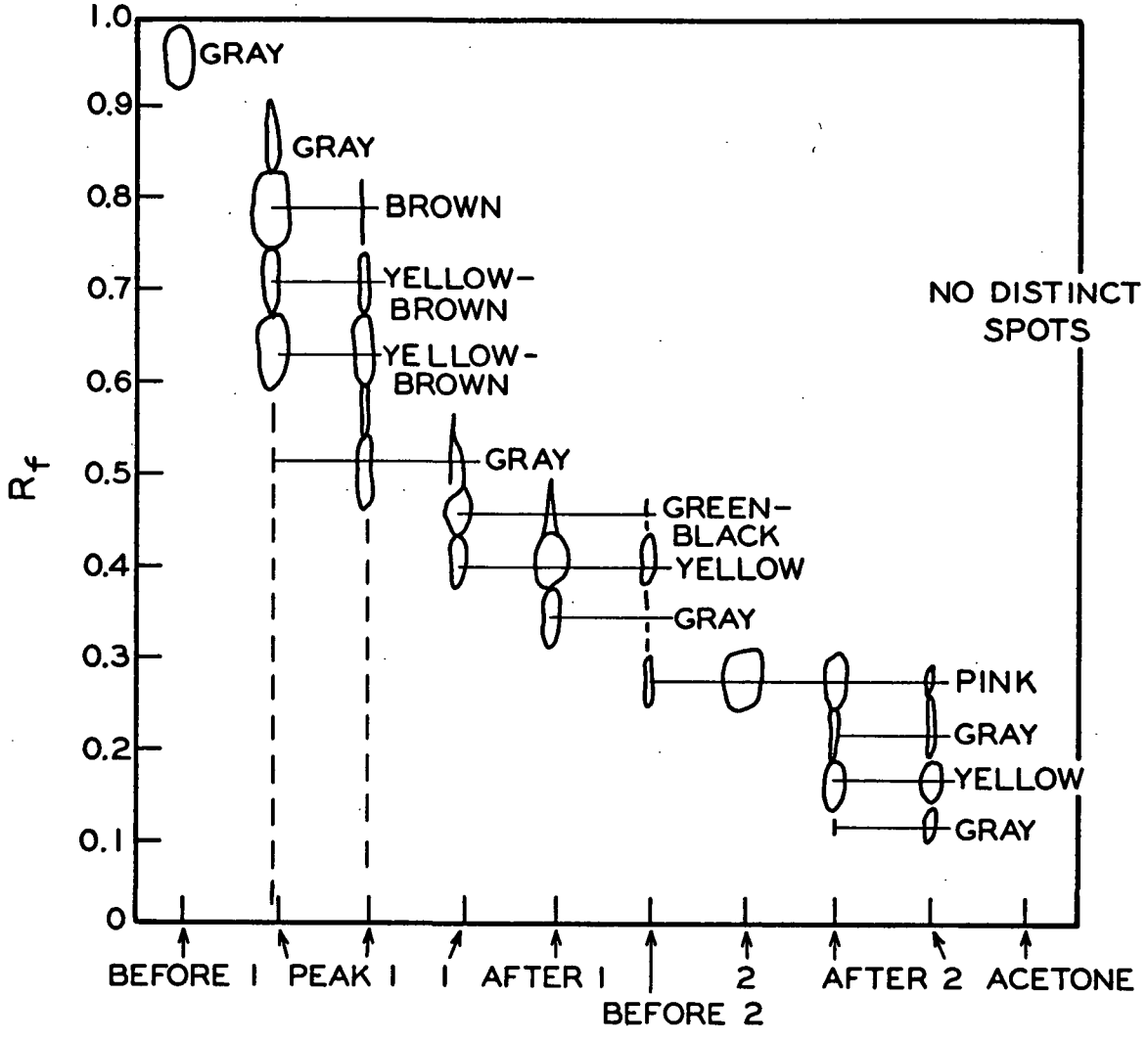

Figure 24. TLC Results of Silica Gel Column Chromatography of Material Recovered from the Lead Filtrate of the Ethyl Acetate Extract

TABLE XII

SUMMARY OF SILICA GEL COLUMN CHROMATOGRAPHY OF MATERIAL REMOVED FROM THE LEAD FILTRATE OF THE ETHYL ACETATE EXTRACT[a]

4:1[b] Eluted, ml.	Weight,[c] mg.	%	Peak	Materials Detected
250	45	3	Before 1	See Fig. 24
75	173	12	1	See Fig. 24
100	256	17	1	Trichocarpin, Salireposide
150	183	13	After 1	Salireposide (25)[d]
150	190	13	Before 2	Salireposide, Salicin
125	477	32	2	Salicin (310)
450	93	6	After 2	Salicin, See Fig. 24
450	27	2	After 2	Salicin, See Fig. 24
300 of acetone	28	2		No distinct materials
Total	1482			

[a] 2.6 g. put on 3.5 x 32 cm. dry-packed, 5:1 silica gel-Celite column.
[b] Eluted with 4:1 chloroform – methanol.
[c] Materials removed from column.
[d] Values in parentheses give mg. of crystals isolated.

MATERIAL RECOVERED FROM THE LEAD PRECIPITATE

The material recovered from the lead precipitate of the ethyl acetate extract, 9.70 g., was put on a 2.6 x 45 cm. polyamide column. The fractions obtained from this chromatogram, summarized in Table XIII, were much different from those obtained from the material recovered from the lead filtrate (Table XI).

TABLE XIII

SUMMARY OF POLYAMIDE COLUMN CHROMATOGRAPHY OF MATERIAL RECOVERED FROM THE LEAD PRECIPITATE OF THE ETHYL ACETATE EXTRACT[a]

H_2O Eluted, ml.	Weight,[b] mg.	%	Peak	Materials Detected
400	1200	13	1	TLC 0.20 yellow-brown, 0.35 brown-orange 0.4-0.6 purple
300	3800	39	2	Pyrocatechol, TLC 0.20, 0.32, 0.46, 0.53 brown, 0.60 blue UV (mild acid hydrolysis-polyamide column chromatography-no new materials)
900	2900	30	3	Trichocarpin, Salireposide (40)[c]
2200	500	5	After 3	Salireposide, TLC 0.42 gray
3000	600	6	4	Trichocarposide with TLC 0.33 brown unidentified material (180)
16000	350	4	After 4	No distinct materials
Ethanol extraction	275	3		No distinct materials
Total	9625			

[a] 9.70 g. put on 2.6 x 45 cm. column.
[b] Material removed from column.
[c] Values in parentheses give mg. of crystals isolated.

A very large amount of material was removed in the first few fractions. TLC showed almost no salicin, although several unidentified materials were present.

Fractions indicated by the second peak contained a large amount of unidentified materials. These fractions were combined and given a mild acid hydrolysis. After removing the sulfuric acid with barium carbonate, the hydrolyzate was chromatographed on another polyamide column. This chromatogram

gave a single peak which contained the same unidentified materials as were present before the mild acid hydrolysis.

Fractions shown by the third peak contained trichocarpin, salireposide, and some other materials. Only the salireposide was obtained as crystals. The trichocarpin was identified by TLC and paper chromatography.

Fractions shown by the fourth peak contained trichocarposide mixed with an unidentified substance. This unidentified substance gave a TLC spot at R_f 0.33. The mixture, as obtained, melted at 184-7°. After several recrystallizations, pure trichocarposide, m.p. 180-2°, was obtained. After the fourth peak, no distinct materials were obtained. The polyamide was removed from the column and extracted with ethanol. No distinct materials were found in the ethanol extract.

In the lead subacetate treatment of Bark 1, the material recovered from the lead precipitate was extracted exhaustively with ethyl acetate and chromatographed on polyamide columns. Almost the same results were obtained as with Bark 2. Trichocarpin, salireposide, some salicin, and a small amount of Compound No. 4 were obtained.

MILD ACID HYDROLYSIS

A portion of the ethyl acetate extract was given a mild acid hydrolysis by dissolving 9.30 g. of extract from Bark 2 in a 0.5N sulfuric acid – 30% ethanol solution and boiling under reflux for one hour. The ethanol was removed by evaporation under reduced pressure and the solution was refrigerated for several days. No crystals were observed. Finally, it was extracted

continuously with ethyl acetate.[1] The 7.4 g. of ethyl acetate extract were placed on a 2.5 x 49 cm. polyamide column and the column was eluted with water.

TLC results from this chromatogram indicated that the separation was poor. Almost all the fractions contained a similar mixture of many materials. Weight results gave a single tailing peak. However, two different crystalline materials were obtained from the chromatogram. The first material, which was obtained from the 1100 to 1500 ml. of eluate, melted at 173-5° and was Compound No. 3. A study of the IR spectrum of Compound No. 3 showed that it had two carbonyl groups and was therefore similar to that of salicyloyl tremuloidin (2-benzoate of salicyloyl salicin). Both spectra are presented in Fig. 25. Only 5 mg. of this material were obtained. An IR spectrum and paper chromatographic results identified the second material as p-coumaric acid. Ten milligrams of acid were obtained from the 5000 to 6000 ml. of eluate.

An attempt was made to obtain a better separation of the materials in this extract by combining most of the fractions and chromatographing this 4.0 g. of material on a 3.5 x 56 cm. polyamide column. Paper chromatography detected salicyl alcohol, pyrocatechol, and p-coumaric acid, but no crystalline materials were obtained.

SUMMARY

The ethyl acetate extract contained a large amount of unidentified material. Some of this was a material which gave salicin after a lead subacetate treatment since some free salicin was present in the extract, but a greater quantity was observed after lead subacetate treatment. Mild acid

[1] Some of the ethyl acetate hydrolyzed in the acidic extraction to give acetic acid and ethanol.

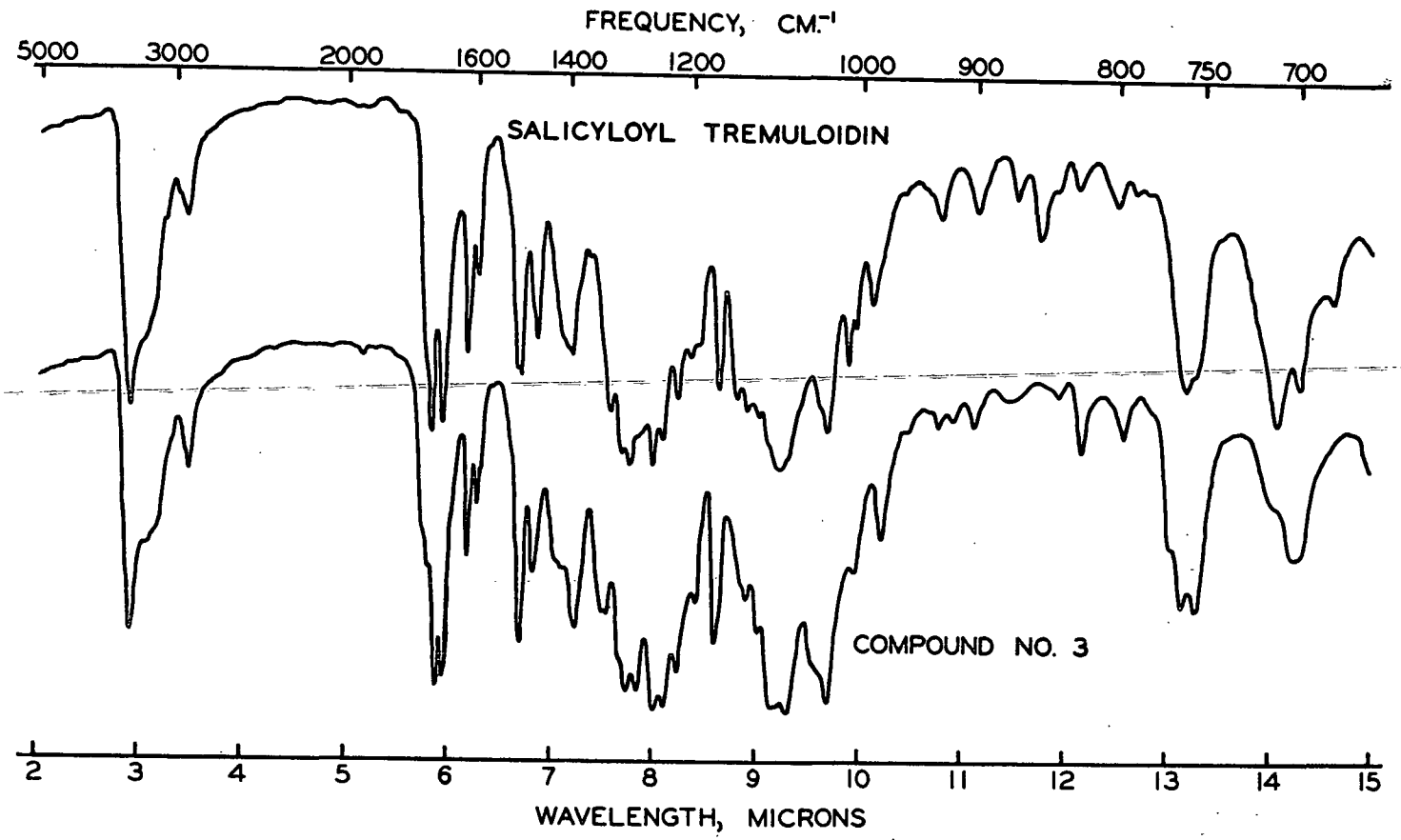

Figure 25. IR Spectra of Compound No. 3 and Salicyloyl Tremuloidin

hydrolysis liberated a very small amount of unidentified material, Compound No. 3, which liberated salicin after mild alkaline hydrolysis. A significant quantity of unidentified p-coumaroyl material, Compound No. 4, also was present in the extract. Salireposide, trichocarpin, p-coumaric acid, trichocarposide, and trace amounts of pyrocatechol, glucose, sucrose, and fructose were detected.

Much of this extract was composed of trace amounts of many materials. These materials gave almost a streak on TLC. This streak was resolved with a silica gel column into a series of short streaks with R_f varying from 0.0 to 1.0.

Most of the ethyl acetate extract was a stable material which did not decompose. The lead subacetate treatment hydrolyzed an unidentified mixture of materials to yield salicin and it separated the extract into two distinct unknown fractions.

Further studies could be made on this extract to determine the structure of the unidentified p-coumaroyl material, Compound No. 4, and to determine the nature of the unidentified materials which gave salicin. In addition, there were large amounts of other unidentified materials which could be studied.

RAFFINATE

INTRODUCTION

A preliminary study of the raffinate was made by direct polyamide column chromatography. In addition, the raffinates were treated with lead subacetate and subjected to preliminary investigations. A flow chart of these procedures is shown in Fig. 26.

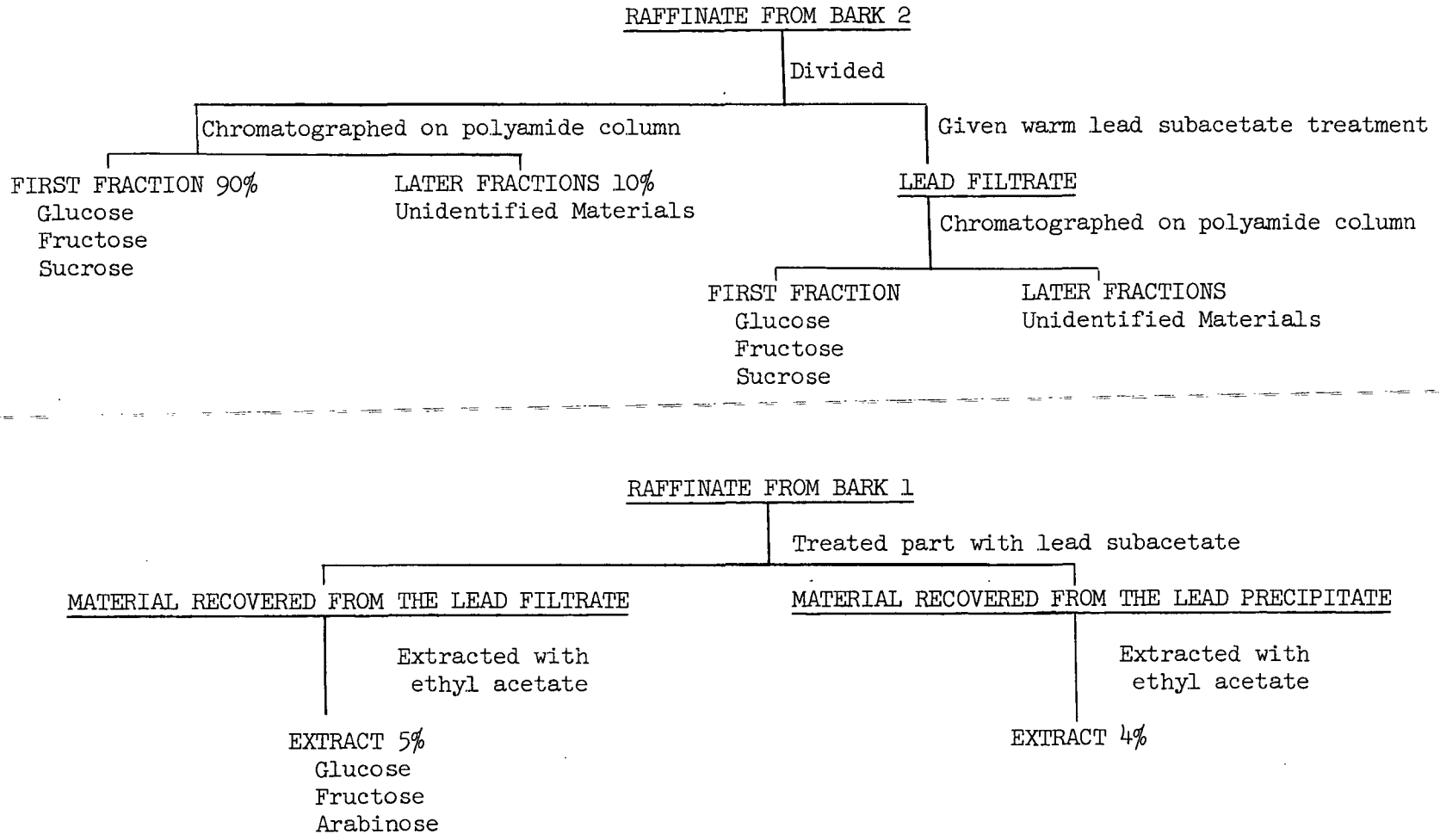

Figure 26. Flow Chart of Processing of Raffinate

POLYAMIDE COLUMN CHROMATOGRAPHY

Part, 11.4 g., of the raffinate was chromatographed on a 2.5 x 32 cm. polyamide column. Elution of the chromatogram with 400 ml. of water removed over 90% of the material. The remaining 10% was removed with four more liters of water. TLC showed only a gray spot at $\underline{R}_f$ 0.0 or a slight streak from 0.0 to 0.4. No colors or distinct spots were observed. Silver and anisidine sprays on paper chromatograms, developed in BPW and EPW, showed spots identical to glucose. Strong fructose and sucrose spots were detected with urea spray. Over 3 g. of gray-white, methanol-insoluble material were obtained from this column chromatogram. This material did not appear to be crystalline and was not investigated further.

LEAD SUBACETATE TREATMENT

Both Bark 1 and Bark 2 raffinates were treated with lead subacetate. For Bark 2, 21.4 g. of raffinate were added to 3 liters of water and 40 g. of lead subacetate and heated for one hour to 55°. The slurry was filtered through Celite and processed in the same manner as the other leaded extracts.

The material recovered from the lead filtrate was concentrated and placed on a 2.5 x 37 cm. polyamide column and eluted with 11 liters of water. Ninety-eight percent of the material removed from the column chromatogram was obtained in the first 500 ml. of eluate. The chromatogram was almost identical with that of the unleaded raffinate.

The Bark 1 raffinate was given a similar lead subacetate treatment (see Fig. 26). Both the materials recovered from the lead filtrate and precipitate were extracted exhaustively with ethyl acetate. The extraction removed less than 10% of the original raffinate. Glucose, fructose, and a trace of arabinose were detected by anisidine and urea sprays on paper chromatograms developed in EPW.

SUMMARY

The preliminary data of this study showed that some glucose, fructose, sucrose, and a trace of arabinose were present in the raffinate. The majority of the raffinate was not identified or extensively investigated. Earlier studies (6) on a similar raffinate of Populus tremuloides and studies by Larson (7) on the raffinate of the brown bark of Populus trichocarpa showed that similar raffinates contained large amounts of complex carbohydrate material such as hemicellulose or pectin. In addition, most soluble salts should be retained in the raffinate. Similar materials probably were the major constituents of this raffinate.

DISCUSSION

INTRODUCTION

The hot water extract of the green bark of Populus trichocarpa was investigated by exhaustive extraction with three neutral organic solvents. Each of these three extracts was separated and investigated by polyamide column chromatography before and after mild lead subacetate treatments. Several glycosides and other materials were obtained from these extracts.

Salicin, trichocarpin, salireposide, a new glycoside – trichocarposide, and trace amounts of tremuloidin and four unidentified materials were obtained. Salicyl alcohol, pyrocatechol, and p-coumaric acid also were present. A semi-quantitative summary of these materials is presented in Table XIV. A more complete summary of the new glycoside, trichocarposide, and the unidentified materials is given in Table XV.

MATERIALS

SALICIN

Salicin is found in most members of the Salicaceae family. It has been found in the lead subacetate treated hot water extract of the bark of Populus trichocarpa (1) and in similarly treated barks and leaves of other Populus species (1, 4, 32). There is some evidence that salicin exists as a polymeric complex which is hydrolyzed by lead subacetate to give salicin (6, 8, 31). However, salicin has been obtained without any alkaline or lead treatments (11-13, 16, 33). This study showed that some salicin was present before the lead treatment, while much more was present after the treatment. Thus, some of the salicin appears to be free and some is part of a more complex material.

TABLE XIV

SEMIQUANTITATIVE SUMMARY OF MATERIALS[a]

Material	% of Chloroform Extract	% of Ethyl Ether Extract	% of Ethyl Acetate Extract	% of Total o.d. Bark
Salicin	T[b] 0.5[d]-8[d] (3)	T - 2[d] (1)[c] T - 2[d] (1)	1-3 (2) 4[d]-11[d](9)	0.08 0.34[d]
Trichocarpin	0.1 -8 (1)	15-39 (35)	1-8 (4)	0.73
Salireposide	T -2.5(1)	2-7 (5)	2.5-11(5)	0.25
Trichocarposide	-	4-9 (7)	1-3 (2)	0.17
Tremuloidin	0.02[d]-0.7(0.1)	-	-	0.001[d]
Compound No. 1	0.18 -4.7 (1)	-	T (0.05)	0.03
Compound No. 2	0.1 -1 (0.5)	-	-	0.01
Compound No. 3	-	-	0.05-? (0.1)	0.003
Compound No. 4	-	-	5-17 (10)	0.32
Salicyl alcohol	2 - 5 (4)	T - 2 (1)	-	0.05
Pyrocatechol	2 - 8 (4)	T - 4 (1)	T	0.05
p-Coumaric acid	-	3-6 (5)	T	0.08
Total	4.9-38(14.6)	24-69 (55)	3.5-50 (30)	2.03

[a] All values from Bark 2.

[b] T = Trace.

[c] Values in parentheses used to calculate % of total o.d. bark.

[d] Quantity obtained after lead subacetate treatment.

TABLE XV

SUMMARY OF NEW MATERIALS

Name	M.P., °C.	TLC	Paper Chromat.[a]	Mild Alkaline Hydrolysis	Enzymatic Hydrolysis	IR Spectra Bands, microns
Trichocarposide	180-2	0.45 Gray Pink	0.45 (0.65) Blue	Salicin p-Coumaric Acid		2.98, 3.47, 5.91, 6.13, 6.23, 6.59, 6.69, 6.87, 7.17, 7.45, 7.53, 7.67, 7.93, 8.07, 8.35, 8.54, 9.29, 9.67, 10.05, 10.23, 11.15, 11.65, 12.09, 12.75, 13.42, 13.64.
Compound No. 1	163-5	0.45 Green Black (Blue UV)	-	Not identified	Glucose	2.99, 3.44, 5.79, 6.20, 6.30, 6.66, 6.86, 7.06, 7.27, 7.64, 7.81, 8.06, 8.27, 8.49, 9.33, 9.56, 9.80, 10.85, 11.16, 11.68, 12.22, 12.78, 13.10, 13.32, 13.64, 14.39.
Compound No. 2	163-6	0.45 Green Black (Blue UV)	-	Not identified	Glucose	2.96, 3.44, 5.91, 6.17, 6.70, 6.87, 7.19, 7.51, 7.79, 8.25, 9.33, 9.82, 11.11, 11.95, 12.43, 12.73, 13.70, 14.44.
Compound No. 3	173-5	0.55 Pink (Blue UV)	0.66 Gray Pink	Salicin Salicylic Acid	-	2.91, 3.48, 5.89, 5.95, 6.18, 6.27, 6.70, 6.83, 7.24, 7.50, 7.55, 7.75, 7.85, 8.02, 8.13, 8.24, 8.42, 8.60, 8.92, 9.03, 9.18, 9.32, 9.70, 9.96, 10.23, 10.48, 10.77, 10.92, 11.12, 11.50, 11.95, 12.17, 12.58, 13.16, 13.30, 14.30, 15.05.
Compound No. 4	170-90	0.35 Pink	0.63 Blue	p-Coumaric Acid	-	3.00, 3.45, 5.90, 6.10, 6.22, 6.58, 6.93, 7.52, 7.75, 7.90, 8.32, 8.56, 9.06, 9.30, 9.75, 11.12, 11.63, 12.07, 12.19, 12.92.
Trichocarpin[b]	134-6	0.45 Green Black (Blue UV)	0.61 Blue	-	Glucose	2.93, 3.43, 5.83, 6.19, 6.29, 6.65, 6.87, 7.26, 7.67, 7.96, 8.07, 8.27, 8.30, 9.32, 9.57, 10.11, 11.19, 11.30, 12.25, 12.80, 13.65, 14.33.

[a] Paper chromatographed with water developer and detected with DPNA.

[b] Not a new material, but put in for comparison with Compounds No. 1 and 2.

Pearl, et al. (6, 8) have shown indirect evidence for a mixture of substances which yield salicin upon mild alkaline treatment. When the hot water extracts from Populus tremuloides and P. grandidentata were treated with lead subacetate, tremuloidin (2-O-benzoyl salicin) and salicin or populin (6-O-benzoyl salicin) and salicin were obtained. When chloroform extracts were given a mild acid hydrolysis, salicyloyl tremuloidin and salicyloyl salicin were obtained.

The mixture of substances, which yield salicin upon mild alkaline treatment, in Populus trichocarpa was not the same as that in P. tremuloides or P. grandidentata. First, large amounts of both tremuloidin and salicin were not found after a mild lead subacetate treatment; only large amounts of salicin were obtained. In the earlier studies on this bark, no tremuloidin was detected and, in this study, a trace of tremuloidin was obtained. However, this trace is insignificant in comparison with the amount of salicin obtained. Second, neither salicyloyl tremuloidin nor salicyloyl salicin was found after a mild acid hydrolysis, but a trace amount of an unidentified material, Compound No. 3, was obtained. A study of the IR spectrum of Compound No. 3 showed that it had two carbonyl groups and some similarities to salicyloyl tremuloidin (see Fig. 25 and Table XV). Finally, the mixture of substances, which yield salicin upon mild alkaline treatment, in Populus tremuloides or P. grandidentata, was extracted with chloroform or ethyl ether, while most of a similar mixture from Populus trichocarpa was not extracted by chloroform or ethyl ether, but by ethyl acetate.

TRICHOCARPIN

Trichocarpin was a major constituent of the bark of Populus trichocarpa comprising over one half of one percent of the ovendry bark. The quantity of

trichocarpin does not change after a lead subacetate treatment. Previously, trichocarpin had been found only in winter twig bark of Populus trichocarpa (14) and in the bark of P. balsamifera (16). Since it has never been detected in many similar studies of Populus tremuloides (3, 5, 6, 32) or P. grandidentata (2, 5) or in the Salix species (9, 11, 13), it appears that trichocarpin may be of taxonomic significance.

Trace amounts of two unidentified materials which were very similar to trichocarpin, Compound No. 1 and Compound No. 2, also were present in the bark (see Table XV). In addition, there were several TLC spots of the same color as trichocarpin indicating that other glycosides with the gentisic acid moiety may have been present in the bark. Similar results were observed by Pearl and Pottenger (16) in their study of Populus balsamifera.

SALIREPOSIDE

Salireposide was present in the bark of Populus trichocarpa. It appears to be present in many of the species of the Salicaceae family (1, 12, 13) and had been found in the previous study of this bark (1). Like trichocarpin, the quantity of salireposide did not change after a mild lead subacetate treatment.

TRICHOCARPOSIDE

A new glycoside, trichocarposide, was found and identified. Trichocarposide, 6-O-p-coumaroyl salicin, was unchanged upon treatment with lead subacetate. Since trichocarposide contains salicin, it could be part of the polymeric complex of salicin which was previously discussed, although there was no evidence to support this premise.

The p-coumaroyl group on trichocarposide is significant. In studies on the alkaline hydrolysis of barks of the Populus genus, p-coumaric acid was the

principal phenolic material isolated from Populus trichocarpa, P. tremuloides, and P. tacamahaca (balsamifera) (34, 35). p-Coumaric acid was also the principal phenolic material obtained after alkaline hydrolysis of the hot water extract of the bark of P. tremuloides (6). These studies suggested that the p-coumaroyl group is combined as an ester, possibly with a glycoside. Yet grandidentatin, found only in Populus grandidentata (2) was the only glycoside isolated from the Populus genus which contains the p-coumaroyl group, although some p-coumaroyl flavonoid glycosides (36-38) and 1-p-coumaroylglucose (27, 30, 36, 39) have been found in other plants. It is now apparent that trichocarposide, a p-coumaroyl glycoside in Populus trichocarpa, accounted for some of the p-coumaric acid found after alkaline hydrolysis of this species.

Another p-coumaroyl material was obtained. This material, Compound No. 4, gave p-coumaric acid after mild alkaline hydrolysis and also would account for some of the p-coumaric acid found after alkaline hydrolysis of the bark.

SALICYL ALCOHOL

A large amount of salicyl alcohol was obtained from the chloroform extract after treatment with lead subacetate. Because of the poor chromatographic separation, it was impossible to determine if all the salicyl alcohol was present before the lead subacetate treatment, although it appeared that it was. This investigation confirms the earlier study (1) that salicyl alcohol is present in the bark of Populus trichocarpa. It would be expected in most species of the Salicaceae family since it is the aglucone of salicin and related glycosides which are almost universally present in this family. Gentisyl alcohol was detected also in the earlier study of this bark (1). Since it is the aglucone of salireposide, this would be expected. In this study, gentisyl alcohol was not isolated, although several paper chromatography spots, which could be weak amounts of this material, were observed.

PYROCATECHOL

Many complex structures of pyrocatechol are found in plants, but pyrocatechol *per se* has been reported with certainty in only a few plants (36). It has been found as a fungistatic material in an earlier study of this bark. Significant amounts of pyrocatechol combined with a large amount of unstable black material, which might be oxidized pyrocatechol, were found in the chloroform extract. After treatment with lead subacetate, most of the pyrocatechol was obtained in the reconstituted precipitate, free of much of the black material. The reduction product of pyrocatechol, *cis* 1,2-cyclohexanediol (4), which was obtained in an earlier study of *Populus trichocarpa* bark (1, 2), was not obtained in this investigation, possibly because the methods of isolation differed. No information about other possible pyrocatechol compounds was obtained.

p-COUMARIC ACID

Free *p*-coumaric acid (*p*-hydroxycinnamic acid) was found in the ethyl ether extract. *p*-Coumaric acid and several other phenolic acids were detected in the previous study of the lead subacetate treated hot water extract of *Populus trichocarpa* bark (1). In the previous study, the presence of the other phenolic acids suggested that the lead subacetate treatment may have caused a hydrolysis. In this study, *p*-coumaric acid was found before any lead subacetate or alkaline treatment and was the only phenolic acid found in significant amounts, indicating that there was no hydrolysis.

Cinnamic acids occur, in combined form, in practically every higher plant (36). Some investigators believe that cinnamic acids, including *p*-coumaric acid, are lignin precursers (40). Thus, any information about cinnamic acid derivatives

will be of importance for the study of lignin precursers as well as biogenetic or taxonomic studies.

Previously, it was explained that p-coumaric acid was the major phenolic material obtained from the alkaline hydrolysis of the green bark of Populus trichocarpa (34). After this hydrolysis, 0.43% of the bark was p-coumaric acid. From this study, it can be calculated that the p-coumaric acid from the free acid, trichocarposide, and Compound No. 4 would be 0.8, 0.06, and 0.10%, respectively, of the bark. Thus, 0.24% of the original 0.43% p-coumaric acid obtained after alkaline hydrolysis has been accounted for. Finding this quantity of p-coumaroyl compounds helps to show why p-coumaric acid is the major phenolic material obtained after alkaline hydrolysis of the green bark of Populus trichocarpa.

OTHER MATERIALS

The raffinate contained glucose, fructose, sucrose, and a trace of arabinose. Glucose and arabinose were found in the raffinate of an earlier study (1) on the bark of Populus trichocarpa, while fructose and sucrose were found in similar raffinates of other Populus species (1, 6). Possible paper chromatographic spots of gentisic acid, p-hydroxybenzoic acid, and quercetin were detected. Gentisic acid is part of the aglycone of trichocarpin and is found in many plants (36). p-Hydroxybenzoic acid is obtained after hydrolysis of this and other barks of the Populus genus (34). Quercetin is obtained from many plants and is found in the Populus species (41-43). Thus, it is not unusual to find any of these materials in this bark.

An attempt was made to detect other known materials listed in Tables I or II. None were found. Since most of the phenolic materials have been obtained after acid or alkaline hydrolysis, it must be assumed that they were present as

a combined form, and, thus, were not detected. There was no evidence for other glycosides, such as have been obtained from other Populus or Salix species (2-13, 44-49). Trace amounts of these glycosides would not be detected by TLC. There were some fluorescent spots on paper chromatograms, which could have been flavonoid materials.

PROCEDURES

LEAD SUBACETATE TREATMENT

This study also provided more information about the mild lead subacetate clarification. It appears that even this mild lead treatment gave some hydrolysis of an unidentified mixture of materials yielding salicin. The other simple phenol glucosides – trichocarpin, salireposide, and trichocarposide – were unchanged by the treatment. When the mild temperature was used, many of the glycosides were found in the precipitate, probably because the material per se was insoluble. About one third of the material was lost in most of the mild lead subacetate treatments. The lost material was not a mixture of all components, but a more specific material since the quantitative results show that all the trichocarpin, salireposide, and trichocarposide was recovered from the combination filtrate and precipitate.

POLYAMIDE COLUMN CHROMATOGRAPHY

Elution chromatography on a polyamide (using Woelm polyamide) column provided a good method of separating the glycosides. Aqueous elution of the column gave simple sugars, salicin, salicyl alcohol, pyrocatechol, tremuloidin, trichocarpin, salireposide, p-coumaric acid, and finally, trichocarposide. Alcohol elution gave no phenyl glucosides, but possibly flavonoid materials and a lot of fatty materials.

EXTRACTIONS

The exhaustive chloroform extraction removed most of the fatty materials, salicyl alcohol, and pyrocatechol.

The exhaustive ethyl ether extraction removed most of the trichocarpin, salireposide, trichocarposide, and p-coumaric acid. Apparently, an exhaustive extraction is required to remove most of these materials since Butin and Loeschcke (14) found trichocarpin in the ethyl acetate extract when they used a similar simple extraction procedure. Furthermore, small amounts of all these materials were obtained from the ethyl acetate extract indicating incomplete extraction.

Finally, the exhaustive ethyl acetate extraction removed the salicin, a complex mixture which yielded salicin after lead subacetate treatment, some materials not completely removed by previous extraction, and many unidentified materials.

Most of the glucose, fructose, sucrose, and arabinose was retained in the raffinate. Other materials, which were soluble in hot water but insoluble in organic solvents, such as soluble salts, pectin, or hemicellulose materials would be in the raffinate.

SUMMARY AND CONCLUSIONS

The hot water extracts of two samples of green bark of Populus trichocarpa were fractionated by exhaustive extraction with chloroform, ethyl ether, and ethyl acetate. Portions of each extract were chromatographed on polyamide columns before and after mild lead subacetate treatments and the eluates were examined for glycosides and related materials. Salicin, trichocarpin, salireposide, tremuloidin, p-coumaric acid, salicyl alcohol, pyrocatechol, and a new glycoside – trichocarposide were isolated. Except for the trace amount of tremuloidin, all materials were obtained from both samples of bark.

A study of the structure of the new glycoside, trichocarposide, was made. A mild alkaline hydrolysis, a carbon and hydrogen analysis, a periodate oxidation, and an enzymatic hydrolysis of trichocarposide showed that trichocarposide is 6-O-p-coumaroyl salicin.

In addition, four other substances of unknown composition were obtained. Two of the unidentified materials were similar to trichocarpin, yet had distinctive IR spectra and higher melting points. The third unidentified material was obtained after a mild acid hydrolysis and appeared to be related to salicyloyl salicin. Whereas the first three unidentified materials were obtained in trace amounts, a significant quantity of the fourth material was obtained in an impure form. p-Coumaric acid was obtained from this fourth unidentified material after a mild alkaline hydrolysis.

The largest glycosidic constituent of the green bark of Populus trichocarpa, trichocarpin, comprised over 0.5% of the o.d. bark. The finding of large quantities of trichocarpin in Populus trichocarpa and P. balsamifera but not in P. tremuloides or P. grandidentata suggest that trichocarpin may be of taxonomic significance.

Quantitative studies showed that while some salicin *per se* was present in the bark, a greater amount was obtained after a lead subacetate treatment. Thus, most of the salicin exists in the bark combined in an unidentified mixture of materials. This unidentified mixture is not the same as that found in *Populus tremuloides* or *P. grandidentata* since large amounts of tremuloidin or salicyoyl tremuloidin were not obtained after hydrolysis and the unidentified mixture was found in the ethyl acetate extract rather than in the chloroform or ethyl ether extract.

The mild lead subacetate treatment did not change significantly the amounts of trichocarpin, salireposide, trichocarposide, or *p*-coumaric acid obtained, although one third of the total material was not recovered after the lead subacetate treatment.

The finding of free *p*-coumaric acid, trichocarposide, and the unidentified material which gave *p*-coumaric acid, Compound No. 4, explains the large amount of *p*-coumaric acid which was previously found after an alkaline hydrolysis of the bark of *Populus trichocarpa*.

The neutral solvent extractions and polyamide column chromatography separated the simple phenol glucosides and many other materials in the hot water extract with no apparent degradation. Many other materials, which were not isolated or identified, were detected by thin-layer and paper chromatography. The detection of these materials shows the need for additional basic studies on the chemical composition of bark.

GLOSSARY

Developers

BPW = 10:3:3 butanol – pyridine – water

EPW = 8:2:1 ethyl acetate – pyridine – water

BFW = 10:4:1 butyl formate – formic acid – water

BFA = benzene saturated with formic acid

BA = butanol saturated with 2% aqueous ammonia

water = water with 1% acetic acid

Sprays

DPNA = diazotized p-nitroaniline followed by sodium carbonate

silver = silver nitrate, sodium hydroxide, sodium thiosulfate, water

anisidine = p-anisidine hydrochloride followed by heating

urea = urea-phosphate followed by heating

indicator = Eastman dye 1954 plus sodium hydroxide to pH 8.9

Other

TLC = thin-layer chromatography

UV = ultraviolet fluorescence

IR = infrared

Bark 1 = Bark obtained in October, 1959

Bark 2 = Bark obtained in March, 1965

raffinate = ethyl acetate raffinate

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